Table S1. shRNAs used to transduce HeLa cells.

Gene name	Accession number	Hairpin number	Source/Clone ID	Target sequence (5′–3′)
RAB11A	NM_004663	H2	TRCN0000073018	CCC TAT AAA CAT AGC AGG ATT
RAB11A	NM_004663	H3	TRCN0000073019	CGA GCT ATA ACA TCA GCA TAT
RAB11A	NM_004663	H4	TRCN0000073020	CCT GTC TCG ATT TAC TCG AAA
RAB11A	NM_004663	H5	TRCN0000073021	GAG CTA TAA CAT CAG CAT ATT
RAB11A	NM_004663	H6	TRCN0000073022	GCC TTA TTG GTT TAT GAC ATT
RAB11B	NM_004218	F1	TRCN0000029184	GCC TTG GAT TCC ACT AAC GTA
RAB11B	NM_004218	F2	TRCN0000029185	CCT ATT CAA AGT GGT GCT CAT
RAB11B	NM_004218	F3	TRCN0000029186	CCA CTA ACG TAG AGG AAG CAT
RAB11B	NM_004218	F4	TRCN0000029187	CGT AGA GGA AGC ATT CAA GAA
RAB11B	NM_004218	F5	TRCN0000029188	CAA GCA CCT GAC CTA TGA GAA
RAB11B	NM_004218	F5	TRCN0000029188	CAA GCA CCT GAC CTA TGA GAA

Table S2. siRNAs used to silence Rab11 effectors.

Gene name	Accession number	Target sequences
RAB11FIP1	NM_001002814	CAAACAGAAGGAAACGAUA GCUAACUGCAGCUUGGGAA AGUGAGAACUUGAACAAUG UCCGCGAGCUGGAAGACUA
RAB11FIP2	NM_001330167	GGAUGAAGGUGAAUUGUGU CGAGCUACCUGGAUUGCUA GGAACAAUAUGACCGCAAG GAUAAGAUGAAGGGUAGAA
MYO5A	NM_000259	GAACAAAUGUGCACUCUUU GAUCAUCUGCUCUGGAUUA AAAGUAAGGUCGUUGCUAA CGCAGGAGGUACAAGAUUA
MYO5B	NM_001080467	GGACUUACCUCUUGGAGAA CAAGUUGGCCUAACAGUGA GCAGAUCUGGCCUACAAUA ACAGUGGCCUUUAUACGAA
SEC8 (EXOC4)	NM_021807	GAAUUGAGCAUAAGCAUGU UAACUGAGUACUUGGAUAU

		GCCGAGUUGUGCAGCGUAA
		ACUGAGUGACCUUCGACUA
SEC10 (EXOC5)	NM_006544	GAAGUCCGAUGCAGAGCAA
		GGAGAUACCUUAUGACACA
		GGAAAGAAUUAGACAGCGU
		CAUUAGGAGUGGAUCGGAA
SEC15A (EXOC6)	NM_019053	GAAGUUUGGUGAAUGGUAU
		GUUGAUGGCUAUAGAAGAU
		GAUAGAGACAGUCGUGAAA
		CCAAACUCCGUGAGGAUAU
SEC15B (EXOC6B)	NM_015189	UAACUGAACUGCUGAAAGU
		GUACUAGUCCGAAGUCUGA
		CAAGUAAGCCACUAUCGAU
		CGGGAAACAUUUGAGAAUU
EXO70 (EXOC7)	NM_001013839	GGUUAAAGGUGACUGAUUA
		GACCUUCGACUCCCUGAUA
		CUAAGCACCUAUAUCUGUA
		CGGAGAAGUACAUCAAGUA
GRAB (RAB3IL1)	NM_013401	GAGAGAAGGGCUCCGAGUU
		GGCCUGGACUUCACGGCAA
		AGAUCAUGAGGUUGCGGAA
		ACAUGAAGCAGGCGGCAUC

Table S3. DNA plasmids used for transfection.

Protein	Plasmid	Source
GFP	pENTR-GFPC1	José Ramalho (Sicgen)
mRab11a	pENTR-GFPC1	José Ramalho (Sicgen)
mRab11b	pENTR-GFPC1	José Ramalho (Sicgen)
mRab11a S25N	pENTR-GFPC1	José Ramalho (Sicgen)
mRab11a Q70L	pENTR-GFPC1	José Ramalho (Sicgen)
mRab11b S25N	pENTR-GFPC1	José Ramalho (Sicgen)
mRab11b Q70L	pENTR-GFPC1	José Ramalho (Sicgen)
mCherry	pCDNA-ENTR-BP-mCherry	José Ramalho (Sicgen)
mRab11a	pCDNA-ENTR-BP-mCherry	José Ramalho (Sicgen)
mRab11b	pCDNA-ENTR-BP-mCherry	José Ramalho (Sicgen)
mRab3a	pENTR-GFPC2	José Ramalho (Sicgen)
rSec15	pJ3 Myc-EGFP	Kind gift of Wei Guo
rSec15	pCDNA3 myc	Kind gift of Wei Guo
hGRAB	pGFP	Kind gift of Mary McCaffrey
hGRAB∆223-228	pGFP	Kind gift of Mary McCaffrey

Table S4. Primers used for qRT-PCR.

Target gene	Sequence
B-ACTIN	Forward 5'-GCAAAGACCTGTACGCCAAC-3'
	Reverse 5'-AGTACTTGCGCTCAGGAGGA-3'
RAB11A	Forward 5'-CGATGGCTGAAAGAACTGAG-3'
	Reverse 5'-GACAGCACTGCACCTTTGGC-3'
RAB11B	Forward 5'-TCACCCGCAACGAGTTCAAC-3'
	Reverse 5'-CTGCACCACGGTAGTACGC-3'
RAB11FIP1	Forward 5'-CAAGGAGCGAGGAGAAATTG-3'
	Reverse 5'-GGTGTCTGACCCACTGTCCT-3'
RAB11FIP2	Forward 5'-TGGGGGATCTGATAGCCCTT-3'
	Reverse 5'-ACTCATATGAAAACTTGAAGATGGC-3'
SEC8	Forward 5'-ATGGCCAGCAAGCACTATCT-3'
	Reverse 5'-AGGTGCCGGTGTAGTTCATC-3'
SEC10	Forward 5'-ATAAAGCAGTGCCAGGAGGG3'
	Reverse 5'-AGCCAGGACTGTTTCTGGATT3'
SEC15A	Forward 5'-GTCACTACACCGGAGCTCAA3'
	Reverse 5'-TGCTCCAGGTGTGTTGTGTT3'
SEC15B	Forward 5'-CTGACCCTGCTTGAGAAGATGA3'

	Reverse 5’-GCCACGGTGTCAATGAGTTTC-3’
EXO70	Forward 5’-CATGGGTTATCAGGGGATTTG-3’
	Reverse 5’-GAGGTCCAGGTGTGGGTAGA-3’
MYO5A	Forward 5’-CAGTGGTCAGAACATGGGTG-3’
	Reverse 5’-TCGCATGGCATACTTAGCTG-3’
MYO5B	Forward 5’-AGAACTGGAGGAGGAGCGAT-3’
	Reverse 5’-GGTTTGATGGGTTCCGCCTA-3’
GRAB	Forward 5’-ATCCGCTACATCCAGCAAGG3’
	Reverse 5’-TAAGCCTCCTGGGGGAAGAA3’

Table S5. Antibodies used for immunoprecipitation and immunoblotting.

Primary antibodies	Host animal	Immunoblotting dilution
Rab11a (Abcam) (ab128913)	Rabbit	1:50,000
Rab11b (Abgent) (AP12943b)	Rabbit	1:1,000
Sec8 (Enzo Life Sciences) (ADI-VAM-SV016-F)	Mouse	1:500
Sec15 (Sigma) (SAB4200612)	Mouse	1:1,000
Exo70 (Milipore) (MABT186)	Mouse	1:1,000
GFP (Sicgen) (AB0020-200)	Goat	1:1,000
mCherry (Sicgen) (AB0040-200)	Goat	1:1,000
GFP (NeuroMab) (clone N86/8)	Mouse	1:3,000
Goat IgG Isotype Control (Invitrogen) (026202)	Goat	-

Rabbit IgG Isotype Control (Sigma) (I5006)	Rabbit	-

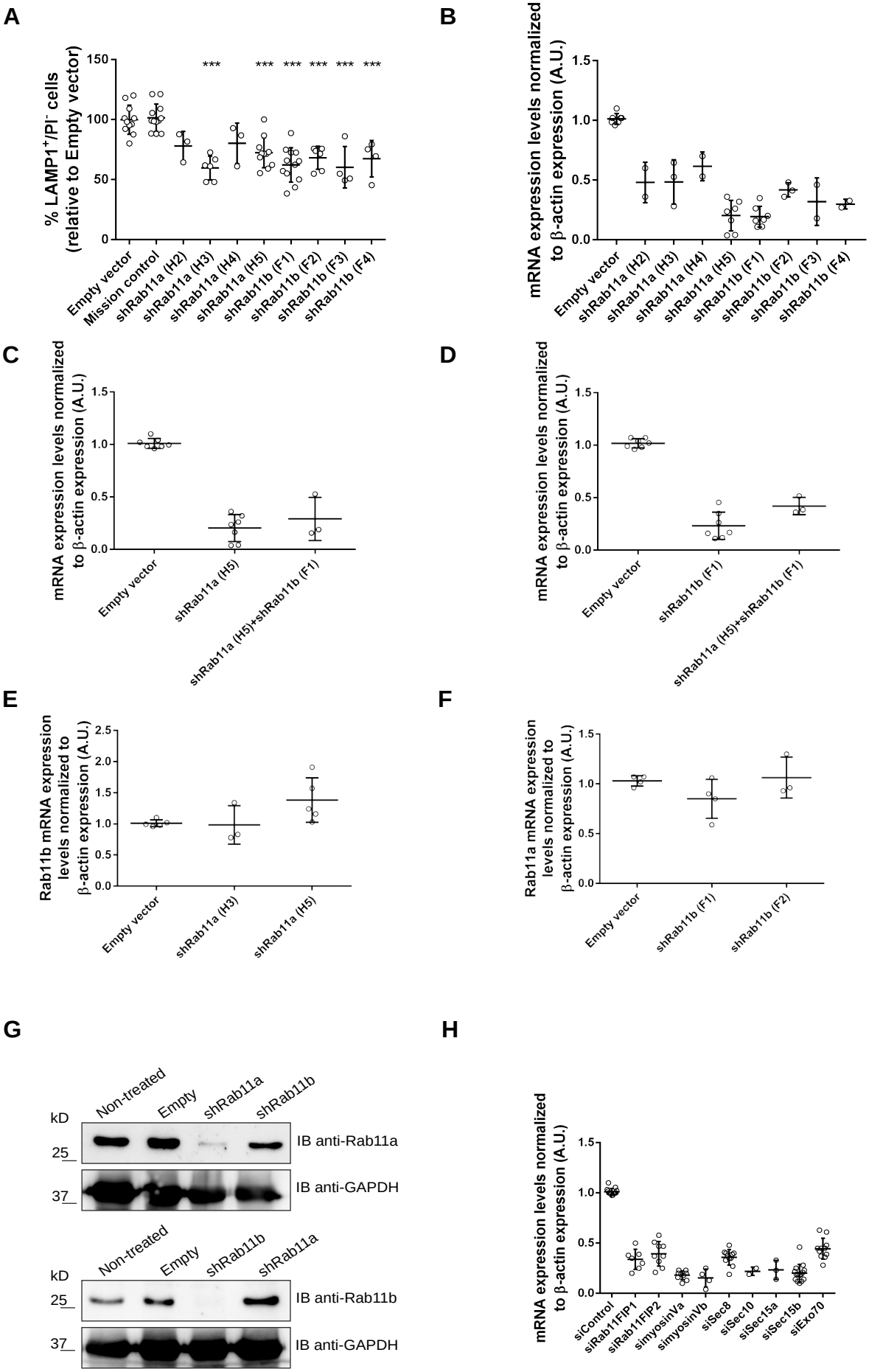


Fig. S1. Silencing efficiency of Rab11a, Rab11b and Rab11 effectors. HeLa cells transduced with lentiviruses encoding different shRNAs targeting Rab11a or Rab11b were selected for 7 days with puromycin. (A) After treatment with 10 µM ionomycin and 4 mM $CaCl_2$ for 10 minutes at 37ºC, to trigger lysosome exocytosis, cells were collected, stained with anti-LAMP1 antibody and analyzed by flow cytometry. Plot represents the percentage of LAMP1-positive cells and PI-negative cells. Cells transduced with an empty vector (Empty) or a non-targeting shRNA (Mission) were used as negative controls. Results were normalized to the empty vector and are represented as mean ± SD of at least three independent experiments. ANOVA followed by Dunnett´s multiple comparisons test was used to compare different data sets with Empty vector (***P<0.001; all others are non-significant). (B) Rab11a and Rab11b silencing efficiency was assessed by qRT-PCR in HeLa cells transduced with lentiviruses encoding different shRNAs targeting Rab11a or Rab11b. Results were normalized for the expression of β-actin. (C) Rab11a expression was analyzed by qRT-PCR, as described before, when silenced alone or in combination with Rab11b. (D) Rab11b expression was analyzed by qRT-PCR, as described before, when silenced individually or in combination with Rab11a. (E) Rab11a expression was analyzed by qRT-PCR, as described before, when Rab11b was silenced with shRNAs targeting Rab11b. (F) Rab11b expression was analyzed by qRT-PCR, as described before, when Rab11a was silenced with shRNAs targeting Rab11a. All results were normalized to the empty vector and are represented as arbitrary units (A.U). Plots represent the mean ± SD of at least three independent experiments. (G) Rab11a and Rab11b protein expression levels were analyzed in HeLa cells transduced with lentiviruses encoding shRNA for Rab11a or Rab11b. Immunoblot was done using rabbit anti-Rab11a or anti-Rab11b antibodies. Non-transduced cells or cells transduced with empty vector were used as controls. Protein levels were normalized to GAPDH. The images are representative of three independent experiments. (H) Rab11 effectors were silenced in HeLa cells as described in Materials and Methods. The relative expression of each silenced gene was analyzed by qRT-PCR and normalized to the expression of β-actin. All results were normalized to siControl and are represented as arbitrary units (A.U). Results are representative of three independent experiments.

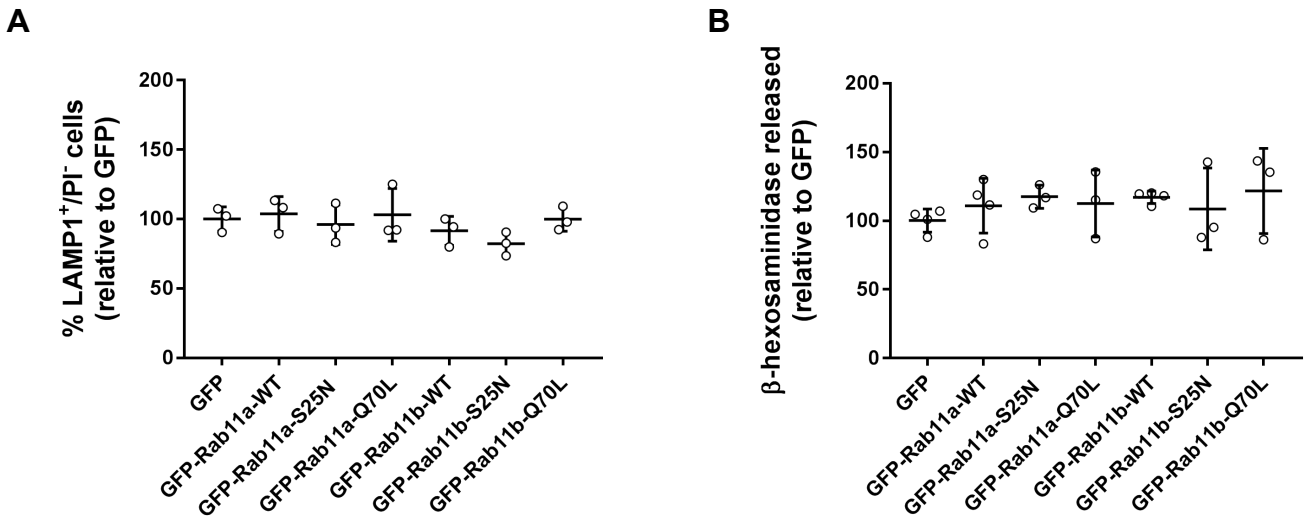


Fig. S2. Overexpression of Rab11a or Rab11b wild-type or mutant forms does not affect LAMP1 cell surface translocation or β-hexosaminidase release. (A) HeLa cells overexpressing the wild-type (WT), dominant negative (S25N) or constitutively active (Q70L) mutants of GFP-Rab11a or -Rab11b were treated with 10 μM ionomycin and 4 mM CaCl2 for 10 minutes at 37ºC, to trigger lysosome exocytosis. Cells were collected, stained with an anti-LAMP1 antibody and analyzed by flow cytometry. Plots represents the percentage of LAMP1-positive cells and PI-negative cells. (B) Cellular extracts and supernatants were collected and β-hexosaminidase release was quantified as described in Materials and Methods. Results were normalized to the cells transfected with a vector encoding GFP alone and are represented as mean ± SD of at least three independent experiments.

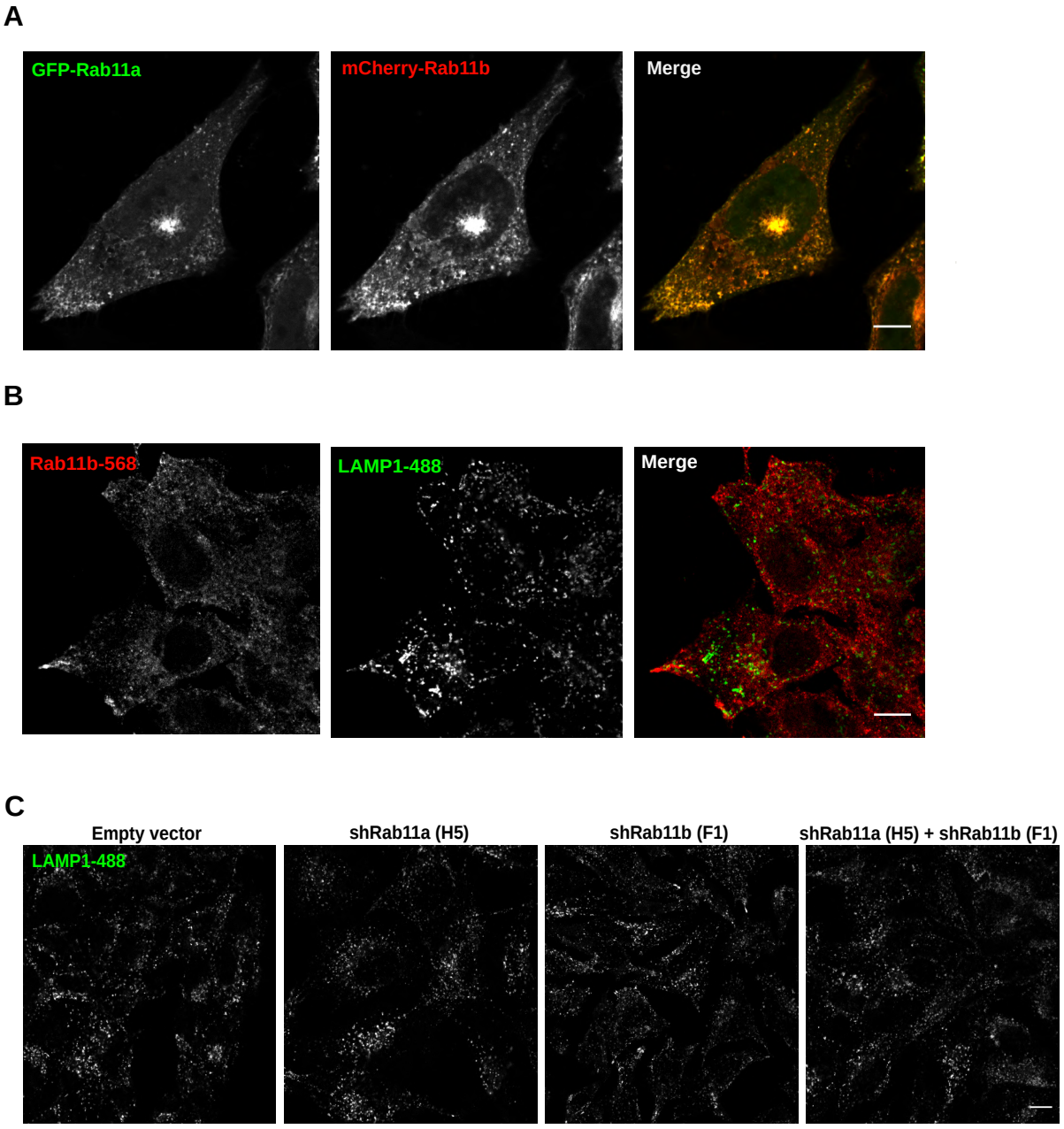


Fig. S3. Rab11a and Rab11b display similar intracellular localization and LAMP1 distribution is not affected upon Rab11a or Rab11b silencing. (A) Representative confocal microscopy images of the localization of Rab11a and Rab11b in HeLa cells transfected with both GFP-Rab11a (green) and mCherry-Rab11b (red) plasmids. Scale bar: 10 µm. (B) Confocal microscopy images of the intracellular localization of Rab11b and LAMP1, in HeLa cells, upon lysosome exocytosis stimulation with 2.5 µM ionomycin and 4 mM CaCl2. Cells were fixed and stained with rabbit anti-Rab11b (red) and mouse anti-LAMP1 (green) antibodies. Scale bar: 10 µm. (C) Confocal microscopy images of the intracellular localization of LAMP1, in HeLa cells transduced with an empty vector (Empty) or lentiviruses encoding shRNAs targeting Rab11a (H5), Rab11b (F1) or both. Scale bar: 20 µm. Results are representative of three independent experiments where at least 15 cells were analyzed in each experiment.

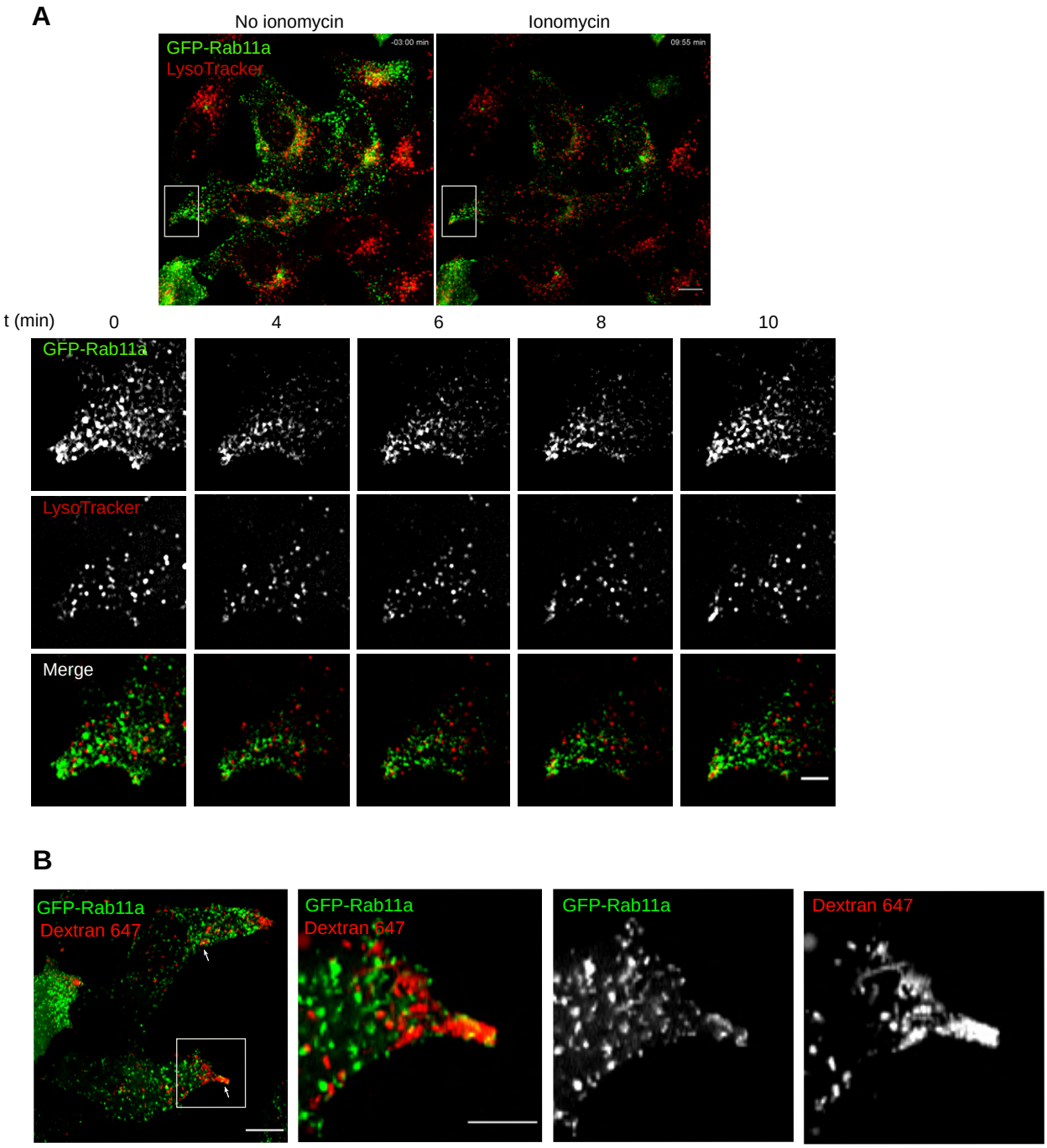


Fig. S4. Colocalization of GFP-Rab11a with lysosomes labeled with dextran. Live cell imaging of HeLa cells transiently transfected with GFP-Rab11a (green). (A) Cells incubated with LysoTracker (red) to label LEs/lysosomes were imaged in a spinning disk confocal microscope for 3 minutes before adding ionomycin and for 10 minutes after ionomycin stimulation. Images were captured every 5-10 seconds. Scale bar: 10 μm. The region outlined with a square was zoomed-in and the channels were split. Different time-frames: 0 (immediately after adding ionomyncin), 4, 6, 8 and 10 minutes are shown. Scale bar: 5 μm. Results are representative of two independent experiments, where at least 15 cells were analyzed in each experiment. (C) Cells were incubated overnight with dextran and chased for 4h. Cells were treated with ionomycin and imaged immediately. White arrows indicate the areas where dextran localize in close proximity with Rab11a-positive vesicles. Scale bar: 20 μm. The selected area was zoomed in. Scale bar: 10 μm. Results are representative of two independent experiments where at least 15 cells were analyzed in each experiment.

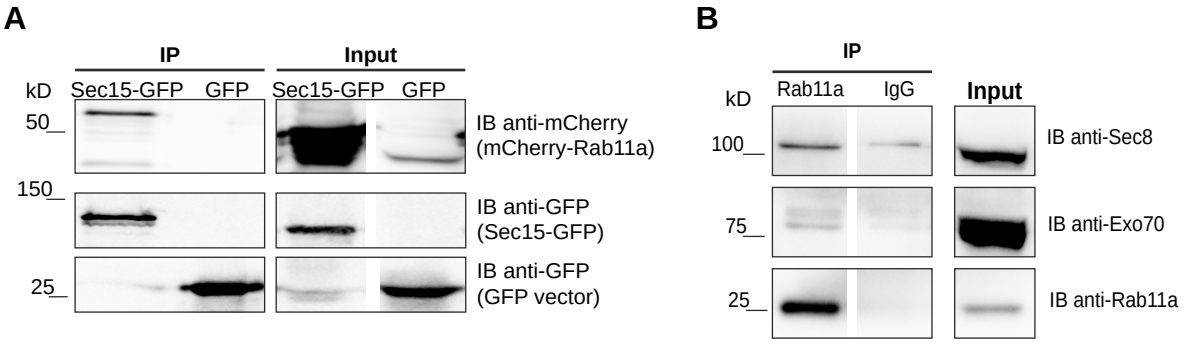


Fig. S5. Rab11a co-immunoprecipitates with different exocyst complex subunits in HeLa cells. (A) Total cell extracts (800 µg) were used to immunoprecipitate Sec15-GFP. Cells expressing GFP were used as a negative control. Immunoblot was done using goat anti-mCherry antibody to detect mCherry-Rab11a or goat anti-GFP antibody. (B) Total cell extracts (700 µg) were used to immunoprecipitate Rab11a, using rabbit anti-Rab11a antibody that recognizes specifically this isoform. Rabbit IgG was used as a negative control. Immunoblot was done using mouse anti-Sec8, mouse anti-Exo70 or rabbit anti-Rab11a antibodies. The images are representative of two or more independent experiments. Inputs correspond to 1/10 of total cell extracts used for immunoprecipitation. Results are representative of three independent experiments.

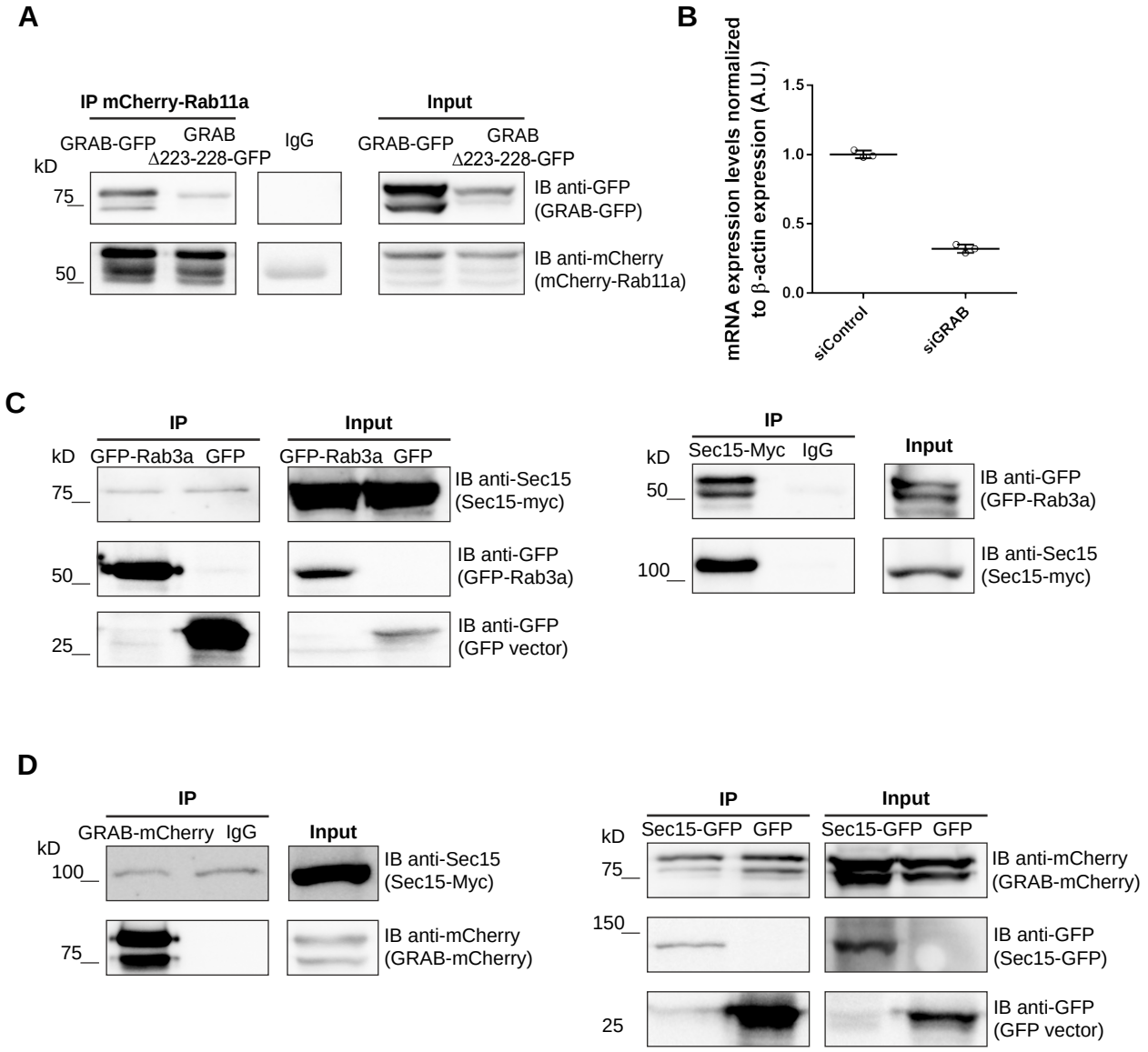
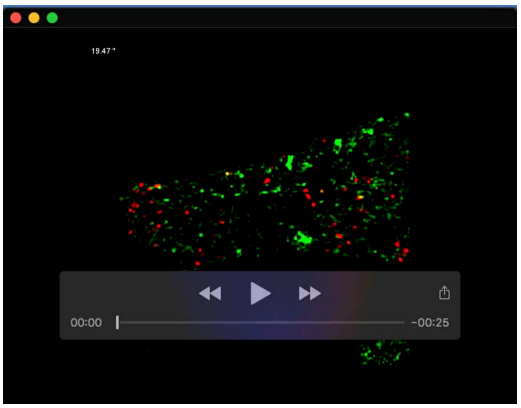
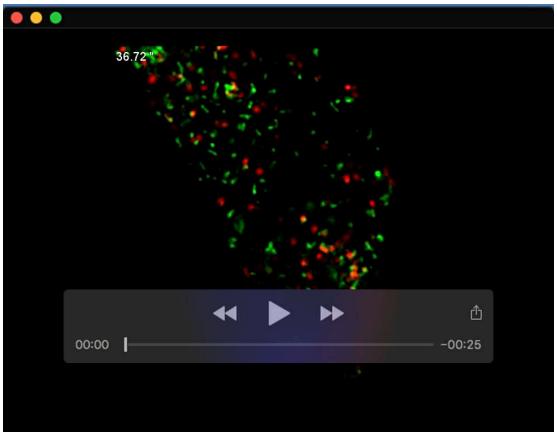


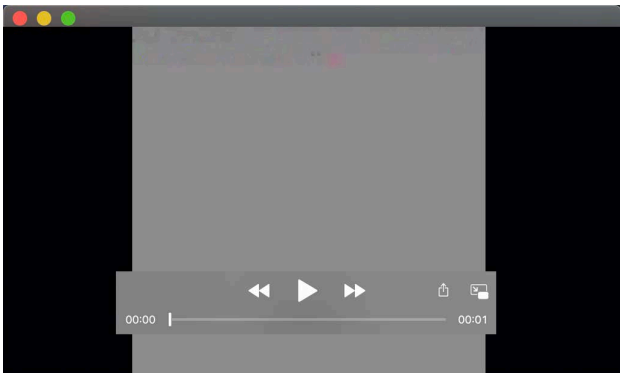
Fig. S6. GRAB interacts with Rab11. (A) Total cell extracts (600 µg) were used to immunoprecipitate mCherry-Rab11a using goat anti-mCherry antibody. Goat IgG was used as a negative control. Immunoblot was done using goat anti-GFP antibody to detect WT GRAB-GFP and GRAB Δ223-GFP. Input corresponds to 1/10 of total cell extracts used for immunoprecipitation. (B) GRAB was silenced in HeLa cells as described in Materials and Methods. Relative mRNA expression was analyzed by qRT-PCR and normalized to the expression of β-actin. Results are represented as arbitrary units (A.U). (C) Total cell extracts (350 µg) were used to immunoprecipitate GFP-Rab3a or Sec15-Myc, respectively. GFP-transfected cells or mouse IgG were used as negative controls. Immunoblot was done using goat anti-GFP or mouse anti-Sec15 antibodies to detect GFP-Rab3a or Sec15-Myc, respectively. Input corresponds to 1/10 of total cell extracts used for immunoprecipitation. (D) Total cell extracts (350-450 µg) were used to immunoprecipitate GRAB-mCherry or Sec15-GFP, respectively. GFP-transfected cells or goat IgG were used as negative controls. Immunoblot was done using goat anti-GFP, goat anti-mCherry or mouse anti-Sec15 antibodies to detect Sec15-GFP, GRAB-mCherry or Sec15-Myc, respectively. Input corresponds to 1/10 of total cell extracts used for immunoprecipitation. Results are representative of three independent experiments.



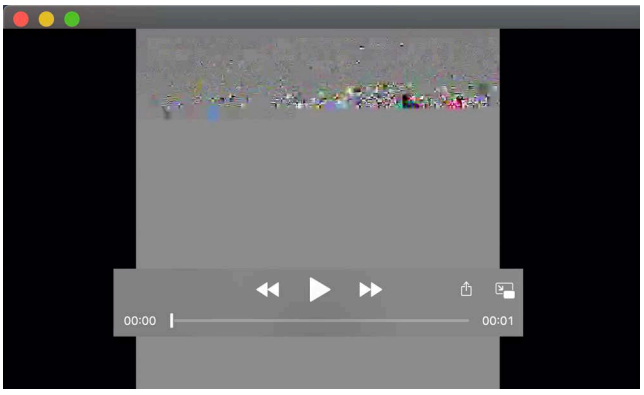
Movie 1. Live cell imaging of HeLa cells transiently transfected with GFP-Rab11a (green) and incubated for 1-2 hours with LysoTracker (red) to label LEs/lysosomes. The cells were imaged in a super-resolution confocal microscope immediately after ionomycin stimulation. Images were captured every 6 seconds for 60 seconds. Results are representative of three independent experiments, where at least 8 cells were analyzed in each experiment.



Movie 2. Live cell imaging of HeLa cells transiently transfected with GFP-Rab11a (green) and incubated for 1-2 hours with LysoTracker (red) to label LEs/lysosomes. The cells were imaged in a super-resolution confocal microscope immediately after ionomycin stimulation. Images were captured every 4 seconds for 90 seconds. Results are representative of three independent experiments, where at least 8 cells were analyzed in each experiment.



Movie 3. Live cell imaging of HeLa cells transiently transfected with GFP-Rab11a (green) and incubated for 1-2 hours with LysoTracker (red) to label lysosomes. The cells were imaged for 3 minutes before adding ionomycin (Time 0) and for 10 minutes after ionomycin stimulation. Images were captured every 5-10 seconds and are representative of two independent experiments, where at least 15 cells were analyzed in each experiment.



Movie 4. Live cell imaging of HeLa cells transiently transfected with GFP-Rab11b (green) and incubated for 1-2 hours with LysoTracker (red) to label lysosomes. The cells were imaged for 3 minutes before adding ionomycin (Time 0) and for 10 minutes after ionomycin stimulation. Images were captured every 5-10 seconds and are representative of two independent experiments, where at least 15 cells were analyzed in each experiment.