LEGENDS OF THE SUPPLEMENTAL FIGURES

Figure S1. Control experiments on the transport of Lamp1 to the cell surface

(A) Lamp1 transport does not depend on late endosomal lipid, LBPA. BHK cells were incubated with 50μg/mL anti-LBPA antibodies for 16h. After stimulation, cell surface Lamp-1 was observed without permeabilization (right panels) as in Fig1, or the endocytosed anti-LBPA antibody was revealed after permeabilization with a secondary fluorescent antibody (left panels). **(B-D)** Lamp1 transport does not require polymerized microtubules. BHK cells were treated or not for 2h with 1μM nocodazole and then stimulated as in Fig 1A. As indicated (B), cell surface Lamp1 was labeled without permeabilization as in Fig 1 or tubulin was immuno-labeled after permeabilization. Bars=10μm. Hexosaminidase release (C) was measured as in Fig 1A and cell surface biotinylation (D) was analyzed as in Fig 1D. C shows the means of 4 experiments +/-SEM. **(E)** Morphology of recycling endosomes after crosslinking with Tfn-HRP. A431 cells were incubated with Tfn-HRP for 15min at 37° C, and then treated without or with DAB-H2O2 as indicated to inactivate the compartment by cross-linking, and processed for electron microscopy. Arrowheads point at tubular electron dense structures, which are not observed in controls, and presumably correspond to Tfn-containing recycling endosomes. Bars=2μm.

Figure S2. Knockdown of the γ1 or μ1 chain of AP1 and of the μ3 chain of AP3

(A-C) The μ1 subunit of AP1 was depleted in A431 with siRNA si#2μ1 against a target sequence different from si#1μ1. After ionomycin stimulation, the release of hexosaminidase (A) and cathepsin D (B) was measured as in Fig 1A and 6A, respectively and cell surface biotinylation (C) was analyzed as in Fig 1D. Panel A shows the mean of 3 experiments +/-SEM.**p<0.025. **(D)** The μ3 subunit of AP3 was depleted with siRNAs against 3 different target sequences (si#1μ3, si#2μ3, si#3μ3), used alone or in combination (triple), and, after ionomycin stimulation, the release of hexosaminidase (A) was measured as in Fig 1A. si#3μ3 is used in Fig 7. **(E)** A431 cells were transfected with siRNAs against the γ1 subunit of AP1 (AP1□□1) and KD efficiency was estimated by western blotting. After stimulation with ionomycin, hexosaminidase release was measured in supernatants as in Fig 1A. AP1□1 KD results (4 experiments) are shown +/- SEM compared to normalized mock controls.

Figure S3. Knock down of clathrin heavy chain

The clathrin heavy chain (CHC) was depleted by RNAi (A). After ionomycin stimulation, the release of hexosaminidase (B) and cathepsin D (C) was measured as in Fig 1A and 6A, respectively. The appearance of Lamp1 on the plasma membrane was visualized by fluorescence microscopy in non-permeabilized cells without or with ionomycin stimulation, and was analyzed after cell surface biotinylation (E) as in Fig 1D. In (B), the means of 3 experiments is shown +/-SEM, **p<0.025.

LEGENDS OF THE SUPPLEMENTAL MOVIES

Movie 1: Rab27a-GFP and Lamp1-cherry after stimulation by live TIRF microscopy. A431 cells were transfected with Rab27a-GFP and Lamp1-cherry 24h before stimulation with 2.5µM ionomycin and imaged during 30s at room temperature with a Zeiss AxioObserver microscope equipped with Zeiss TIRF slider module and HQ2 Camera from Photometrics. Exposure time was 200ms. The movie illustrates the occurrence of fusion, as shown in the snapshots of Fig 5B.

Movie 2. Lamp1-GFP by live confocal microscopy. Lamp1-GFP transfected BHK cells were visualized with LSM510 Zeiss confocal microscope in a CO_2 chamber at 37° C during 3min (each frame: 6s 30). The Z position was changed by hand (at 100s) to observe perinuclear labeling at the equatorial level of the cells (nucleus is clearly visible), or high overall Lamp1 mobility (basal surface of cells without visible nucleus).

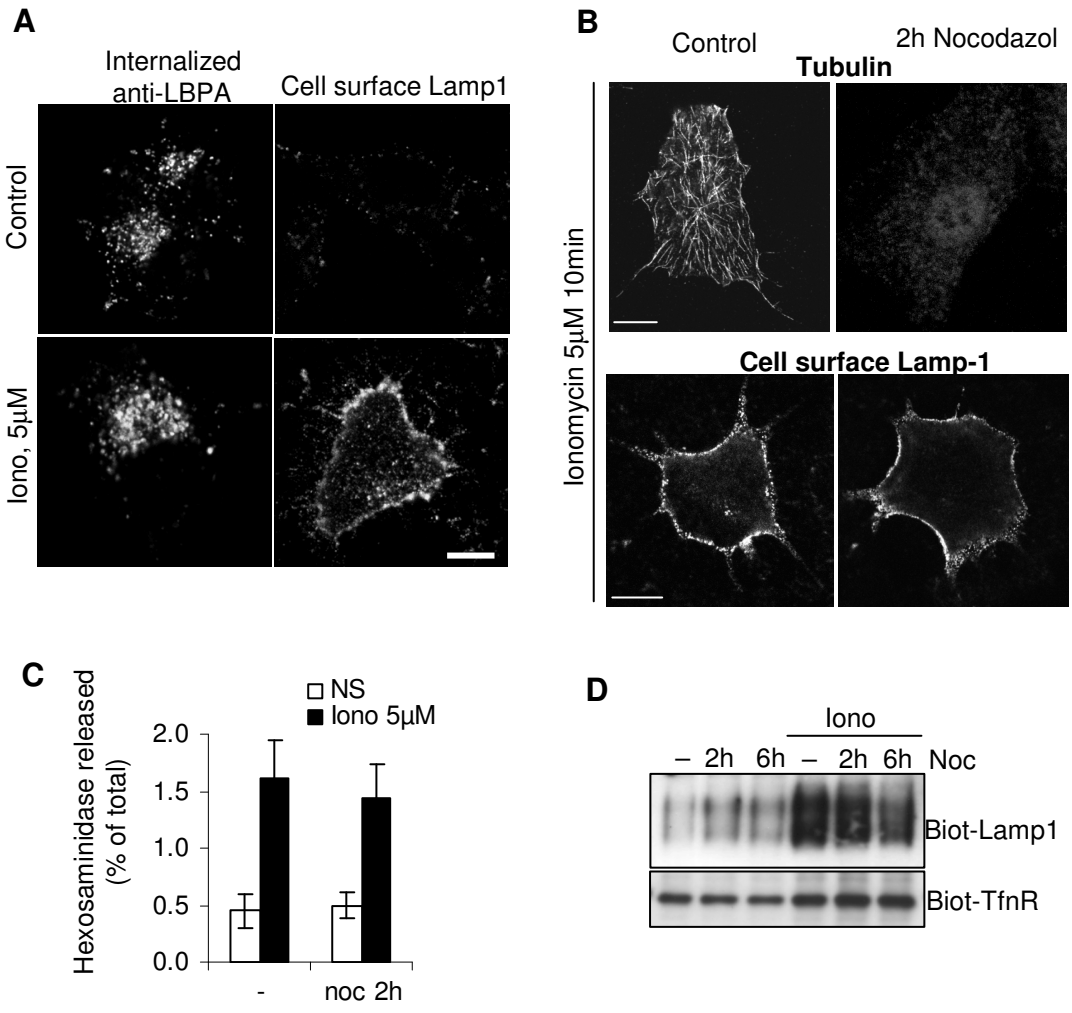
Movie 3. Lamp1-GFP in U18666A-treated cells by live confocal microscopy. Cells expressing Lamp1-GFP were treated overnight with U18666A to induce cholesterol accumulation in Lamp1-containing late endocytic compartments (see also Fig 2). Cells were imaged during 7min (equatorial section).

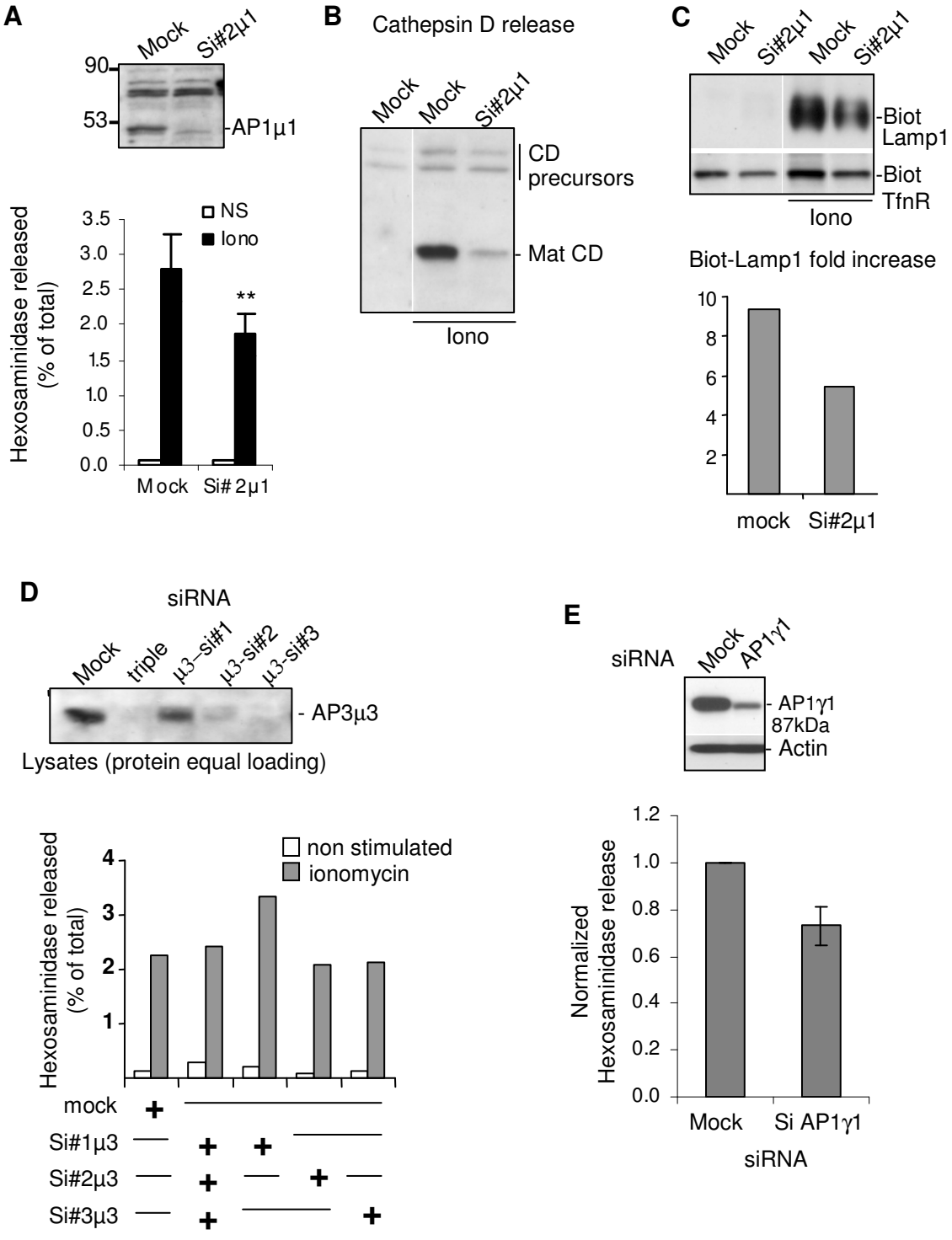
Movie 4. Rab27a-GFP and Lamp1-cherry in U18666A-treated cells by live TIRF microscopy. Cells transfected with Rab27a-GFP and Lamp1-cherry were treated overnight with U18666A and imaged by TIRF as in Movie1. Snapshots of the boxed area are shown in Fig 5C.

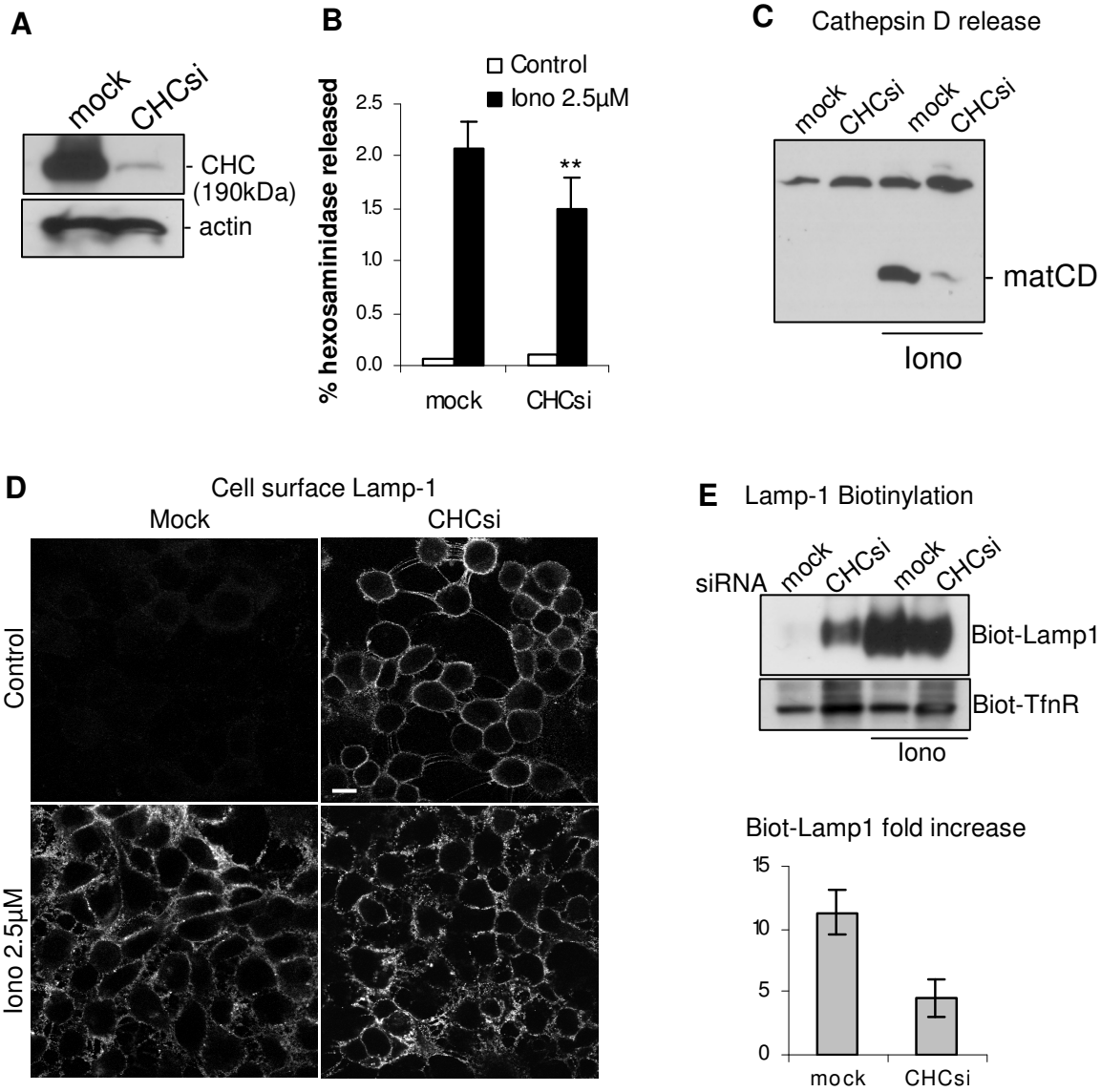
Movie 5. Rab27a-GFP and Lamp1-cherry by live TIRF microscopy.

The experiments were as in Movie1 but without ionomycin stimulation. Snapshots of the area boxed in the first frame are shown in Fig 5A.

Movie 6. Gadkin-GFP and Lamp1-cherry by live TIRF microscopy. A431 cells transfected with Gadkin-GFP and Lamp1-Cherry were imaged as in Movie1 but without ionomycin. Snapshots are shown in Fig 8B.


A
Internalized anti-LBPA
Cell surface Lamp1
Control
Iono, 5µM
B
Control
2h Nocodazol
Tubulin
Ionomycin 5µM 10min
Cell surface Lamp-1
C
NS
Iono 5µM
Hexosaminidase released (% of total)
2.0
1.5
1.0
0.5
0.0
-
noc 2h
D
Iono
– 2h 6h – 2h 6h Noc
Biot-Lamp1
Biot-TfnR
E
Tfn-HRP 15min NoDAB
Tfn-HRP 15min +DAB H2O2


A
Mock
Si#2µ1
90
53
AP1µ1
Hexosaminidase released (% of total)
NS
Iono
**
Mock
Si# 2µ1
B
Cathepsin D release
Mock
Mock
Si#2µ1
CD precursors
Mat CD
Iono
C
Mock
Si#2µ1
Mock
Si#2µ1
Biot Lamp1
Biot TfnR
Iono
Biot-Lamp1 fold increase
mock
Si#2µ1
D
siRNA
Mock
triple
µ3–si#1
µ3-si#2
µ3-si#3
- AP3µ3
Lysates (protein equal loading)
non stimulated
ionomycin
Hexosaminidase released (% of total)
mock
Si#1µ3
Si#2µ3
Si#3µ3
E
siRNA
Mock
AP1γ1
- AP1γ1 87kDa
- Actin
Normalized Hexosaminidase release
Mock
Si AP1γ1
siRNA


A
mock
CHCsi
- CHC (190kDa)
- actin
B
Control
Iono 2.5µM
% hexosaminidase released
2.5
2.0
1.5
1.0
0.5
0.0
**
mock
CHCsi
C
Cathepsin D release
mock
CHCsi
mock
CHCsi
- matCD
Iono
D
Cell surface Lamp-1
Mock
CHCsi
Control
Iono 2.5µM
E
Lamp-1 Biotinylation
siRNA
mock
CHCsi
mock
CHCsi
Biot-Lamp1
Biot-TfnR
Iono
Biot-Lamp1 fold increase
15
10
5
0
mock
CHCsi