SUPPORTING APPENDIX

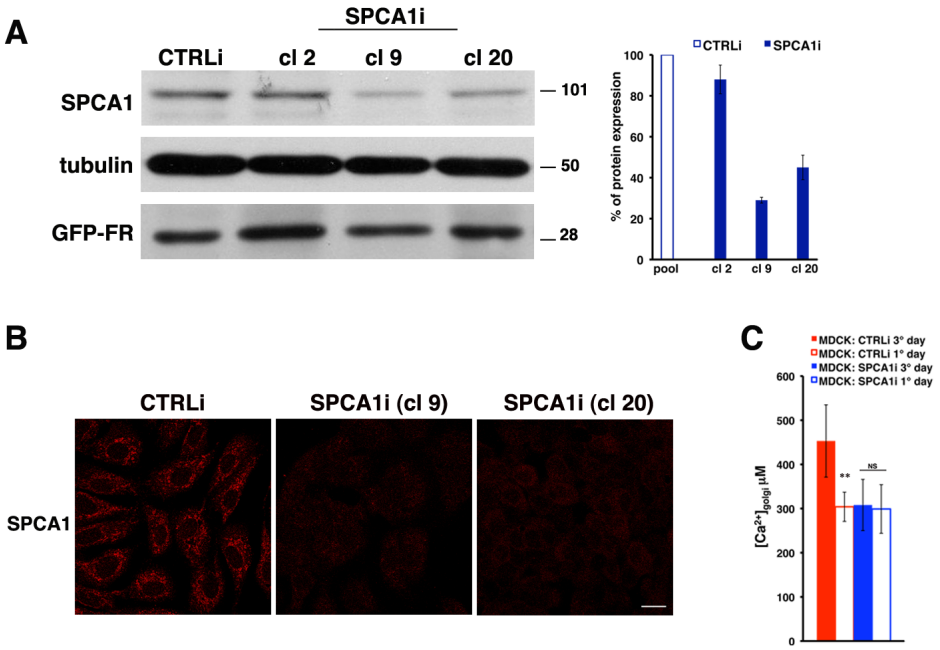

Figure S1: **Generation of MDCK GFP-FR cell line stably silenced for SPCA1**

(A) The levels of SPCA1 expression in MDCK GFP-FR stably interfered with scrambled (CTRLi) or specific short hairpin RNA (SPCA1i) were tested by Western blotting. Tubulin was used as loading control. Note that the transfection of shRNAs did not affect the expression of GFP-FR. Densitometric analyses of three different experiments are shown. Results show the percentage of SPCA1 expression in SPCA1i clones compared with scrambled interfered cells (set equal to 100%). (B) CTRLi and SPCA1i MDCK cells were stained with SPCA1 and revealed wit Alexa-546 antibodies. Scale bar, 10 μm. (C) Golgi [Ca^{2}] quantification in MDCK:GFP-FR cells control-interfered (CTRLi) or SPCA1-interfered (SPCA1i) cells, in fully polarized (3° day) and non-polarized (1° day) conditions, were measured following the same procedure described in Figure 1C. The data represent the mean of four (for polarized conditions) and three (for unpolarized condition) independent experiments performed in two knockdown clones.

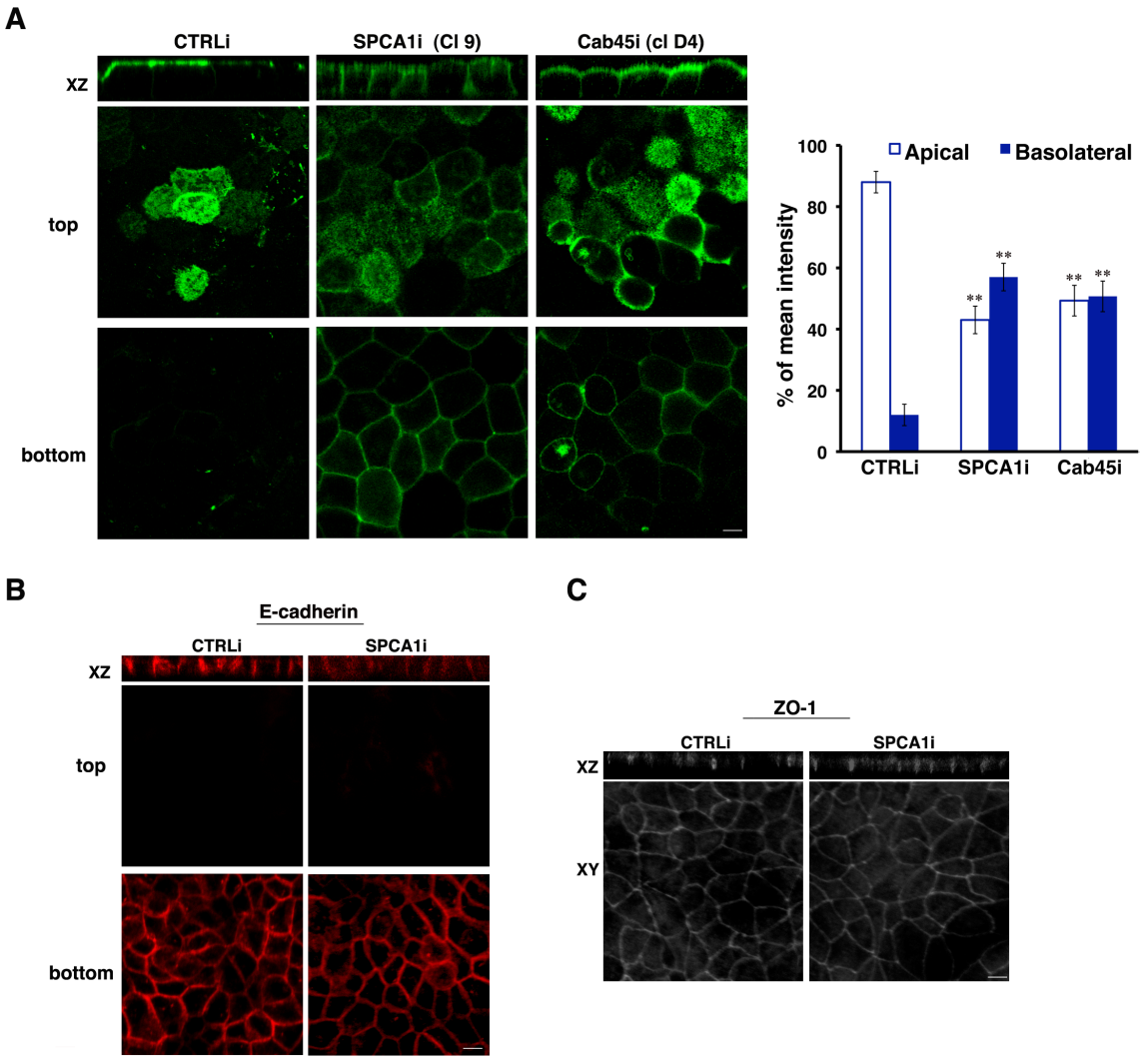

Figure S2: SPCA1 knockdown does not alter apical GFP-FR distribution, epithelial polarity or apical and basolateral localization of endogenous proteins.

(A) MDCK GFP-FR CTRLi, SPCA1i or Cab45i were grown for 4 days on filter were imaged in live conditions by using confocal microscopy. Mean fluorescence intensities at the apical and basolateral surface were measured and expressed as percentages of the total fluorescence. Scale bar, 6 μm. Error bars, ± SD; ** $p<0.01$; Student's *t*-test. (B, C) MDCK CTRLi and SPCA1i cells were grown on filters for 4 days and then processed for immunofluorescence using specific antibody against E-cadherin (B, left) and ZO-1 (C). Scale bars, 6 μm.

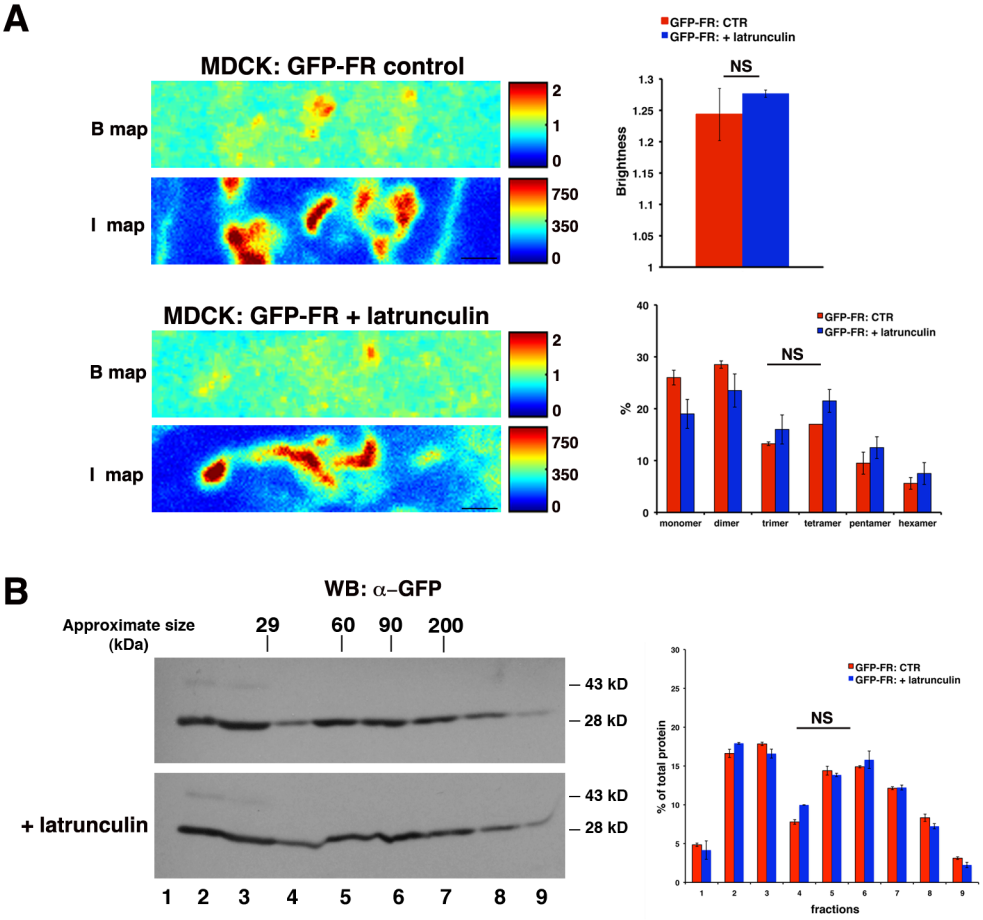
A
MDCK: GFP-FR control
B map
I map
2
1
0
750
350
0
GFP-FR: CTR
GFP-FR: + latrunculin
NS
Brightness
1.3
1.25
1.2
1.15
1.1
1.05
1
MDCK: GFP-FR + latrunculin
B map
I map
2
1
0
750
350
0
GFP-FR: CTR
GFP-FR: + latrunculin
NS
%
40
30
20
10
0
monomer
dimer
trimer
tetramer
pentamer
hexamer
B
WB: α–GFP
Approximate size (kDa)
29
60
90
200
– 43 kD
– 28 kD
+ latrunculin
– 43 kD
– 28 kD
1 2 3 4 5 6 7 8 9
GFP-FR: CTR
GFP-FR: + latrunculin
NS
% of total protein
fractions
C
control
+ latrunculin
XZ
top
bottom
Apical
Basolateral
NS
% of mean intensity
100
80
60
40
20
0
CTRL
+ latrunculin

Figure S3: Alteration of actin dynamics does not affect GPI-AP Golgi homocluster organization and apical sorting in MDCK cells

(A) After 4 days in culture, polarized MDCK cells expressing GFP-FR were treated or not with latrunculin and imaged *in vivo* for N&B at the level of the Golgi complex. Representative B and I maps are shown on the left. Scale bars, 0.9 μm. On the right upper panel, quantification of the brightness of GFP-FR from three independent experiments is plotted. On the right lower panel, graphical representation of the percentage of pixels falling into the different classes of B values (from monomer to hexamer) on the basis of the calibration curve (1). Values are expressed as the mean of three independent experiments, n>25 cells. Error bars, ± SD. (B) In order to analyze exclusively the GFP-FR Golgi pool, MDCK cells were grown on filters and were treated with trypsin (25 μg/ml for 25 min) exclusively at the apical side in order to remove the pool of GFP-FR already present at the plasma membrane and purified on a velocity gradient. Cells were lysed and run on a velocity gradient as described in methods. Fractions were collected from the top (Fraction 1) to the bottom (Fraction 9), TCA-precipitated, run on a SDS-PAGE gel and revealed by western blotting with a specific anti-GFP antibody. Molecular weight markers are indicated on the top of the panels. The molecular weight of the monomeric form of GFP-FR is indicated together with the band at 43 kDa, which represents a partially denatured dimer of GFP. On the right panel, the distribution of GFP-FR in the fractions of the gradient is expressed as a percentage of the total protein. Mean values of two independent experiments are shown. (C) MDCK GFP-FR cells, grown on filters, were treated with trypsin as in (B), then incubated or not with latrunculin for 30 min and imaged in live conditions by using confocal microscopy. Mean fluorescence intensities on the apical and basolateral surface were measured and expressed as percentages of the total fluorescence. Scale bar, 6 μm.

Error bars, ± SD. NS, not significant.

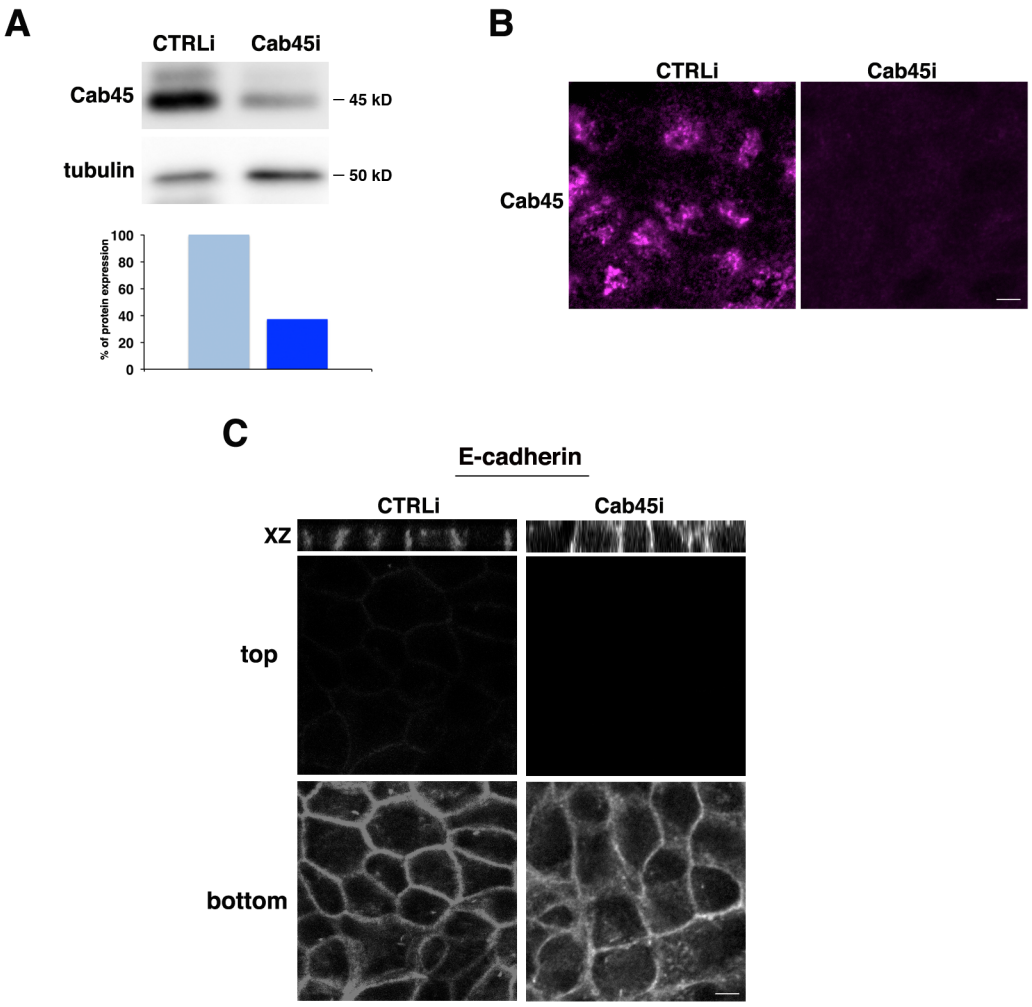

Figure S4: Cab45 knockdown does not alter apical and basolateral localization of endogenous proteins

(A) The levels of Cab45 expression in MDCK GFP-FR stably interfered with scrambled (CTRLi) or specific short hairpin RNA (Cab45i) was tested by Western blotting. Tubulin was used as loading control. Densitometric analyses of three different experiments are shown. Results show the percentage of Cab45 expression in Cab45i cells compared with scrambled interfered cells (set equal to 100%). (B) CTRLi and Cab45i MDCK cells were stained with Cab45 and revealed with Alexa-633 antibodies. Scale bar, 6 μm. (C) CTRLi and Cab45i MDCK cells, grown on filters for 4 days, were subjected to immunofluorescence assay using E-cadherin antibody. Scale bars, 6 μm.

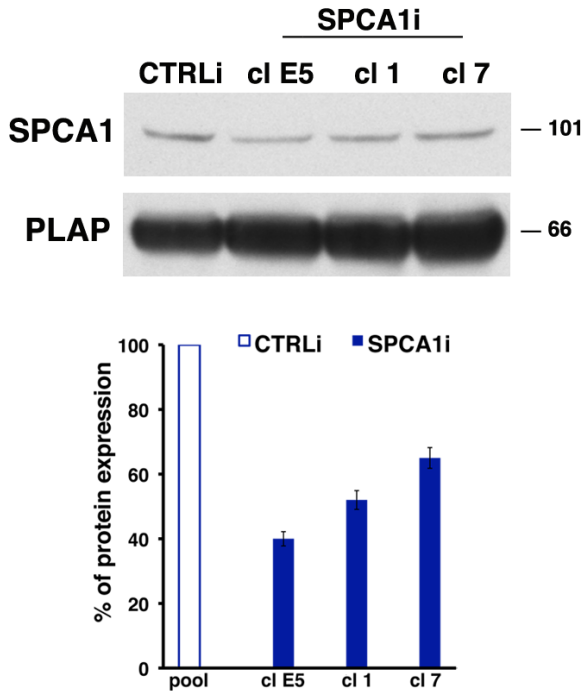

Figure S5. Generation of MDCK PLAP cell line stably silenced for SPCA1

(A) The levels of SPCA1 expression in MDCK PLAP stably interfered with scrambled (CTRLi) or specific short hairpin RNA (SPCA1i) were tested by Western blotting. The expression levels of PLAP are also shown. Densitometric analyses of two different experiments are shown; results show the percentage of SPCA1 expression in SPCA1i clones compared with scrambled interfered cells (set equal to 100%).

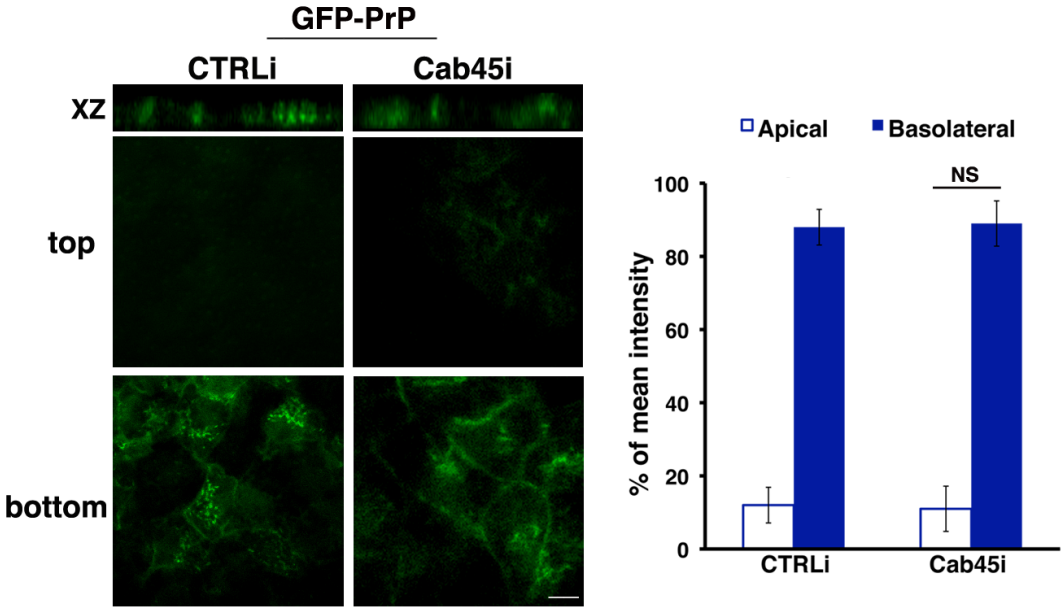

Figure S6: Cab45 knockdown does not alter basolateral localization of GFP-PrP

MDCK GFP-PrP CTRLi or Cab45i were grown for 4 days and then fixed and imaged by using confocal microscopy. Mean fluorescence intensities at the apical and basolateral surface were measured and expressed as percentages of the total fluorescence. Scale bar, 6 μm. Error bars, ± SD; NS, not significant; Student's *t*-test.

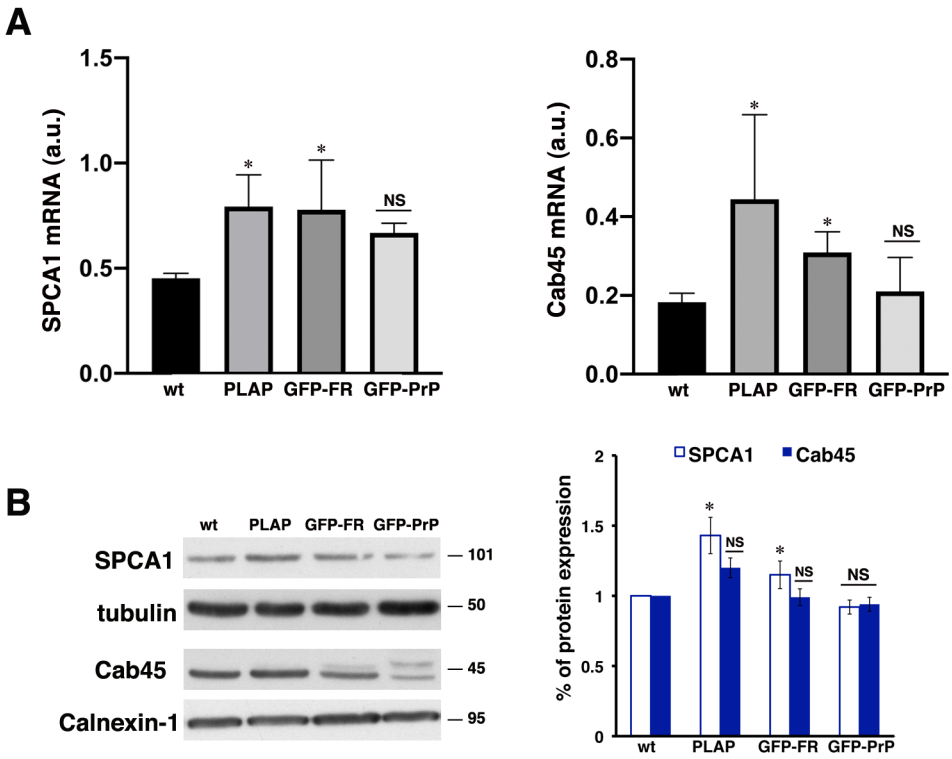

Figure S7: The expression of apical GPI-APs correlates with an increase of mRNA of SPCA1 and Cab45

(A) SPCA1 and Cab45 mRNA levels in MDCK wild-type (wt) and stably expressing GPI-APs (PLAP, GFP-FR and GFP-PrP), grown for 3 days, were analysed by RT-qPCR and normalized to HPRT and Ubch5 mRNA levels; experiments were performed 3 independent times. (B) SPCA1 and Cab45 protein levels in MDCK wild-type and stably expressing the aforementioned proteins, grown for 3 days, were tested by Western blotting. Tubulin and calnexin-1 were used as loading control. Densitometric analyses of two different experiments are shown. Results show the amount of SPCA1 and Cab45 expression in the different MDCK cells expressing GPI-APs compared with wild-type cells (set equal to 1). Error bars, ± SD; NS, not significant; * $p<0.05$, Student's *t*-test.

Materials and Methods

Gene silencing and antibodies

RNA interference was obtained by transfecting specific short hairpin RNAs (from Open Biosystems) bearing this targeting sequence: sh-1 ACCATTGTGCGTGAAGGAAA; sh-2 GAGGCCTTAATTGCTCTTGCAAT. As a negative control, we used an shRNA against GFP, GGCACAAGCTGGAGTACAACTA. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Stably transfected cells were obtained after selection with puromycin (1.5 μg/ml, Sigma).

Cab45 knockdown was performed by using a lentiviral vector pRFP-CB-sh sequence TGTGAATACTGACCGGAAGATAAGCGCCA (Origene); as a negative control a non-effective 29-mer scrambled shRNA cassette in the same p-RFP-CB-sh Lenti Vector (ref. TR30033) was used. MDCK cells were infected for 24h with lentiviral particles and then stable clones were collected after selection with blasticidin. Screening of positive clones was carried out by analyzing RFP fluorescence.

We used the following antibodies: polyclonal anti-GFP (Clontech), polyclonal anti-PLAP (from Rockland), polyclonal anti-giantin (from Ozyme), polyclonal anti-furin convertase (from Thermofisher), monoclonal anti-ATP2C1 (from Biorad) and polyclonal elF4a (from Cell Signaling); monoclonal anti-TGN46 (from ABD Serotec); polyclonal Cab45 from J.V. Blume laboratory.

Perturbation of actin cytoskeleton

To perturb the actin cytoskeleton we incubated cells in culture medium at 37°C with 6 μM latrunculin A (Molecular Probes) for 5 minutes as previously described (3).

Perturbation of cellular calcium content

To perturb the cellular calcium we used the calcium ionophore ionomycin (39, 40). Cells were incubated for 40 min in modified Krebs-Ringer buffer (135 mM NaCl, 5 mM KCl, 1 mM K_2PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4) supplemented with ionomycin (5 μM) and 600 μM EGTA.

Deglycosylation assay

Deglycosylation of protein extracts was carried out by using Protein Deglycosylation Mix II kit (New England biolabs) according to the manufacturer's protocol.

Velocity gradients

Purification of proteins on velocity gradients is a biochemical method that allows purifying proteins according to their molecular weight, independently of their association with membrane domains as previously described (4, 79, 80). Briefly, cells were lysed in 20 mM Tris, pH 7.4, 100 mM NaCl, 1% TX-100 (with or without 0.4% SDS). Lysates were scraped, sheared through a 26-g needle and layered on top of a discontinuous sucrose gradient (30-5%) or a glycerol gradient (20-40%) in the same buffer containing 0.1% TX-100. After centrifugation at 45,000 rpm for 16 h in a Beckman SW50 ultracentrifuge, fractions of 500 µl (for 5-30% gradient) and 300 µl (for 20-30% gradient) were harvested from the top of the gradient.

Temperature block

To achieve an almost complete protein block in the TGN, we used a previously published protocol (2, 47). Filter-grown cells were incubated at 19.5°C for 2 h in areal medium (F12 Coon's modified medium without NaHCO3 and with 0.2% BSA and 20 mM Hepes, pH 7.4). In the last hour at 19.5°C, they were treated with 150 µg/ml cycloheximide.

RT-qPCR

Total RNA extraction from the different MDCK cell lines was performed using the RNeasy Mini Kit from Qiagen. Reverse transcription was done using the Biorad iScript gDNA Clear cDNA Synthesis Kit. Oligonucleotides were designed using Prime PCR Look Up Tool (Bio-Rad) and purchased from Eurofins Genomics. Quantitative PCR was performed using the Bio-Rad iTaqTM universal SYBR® Green supermix and analysed using a CFX96TM real-time PCR detection system under the CFX Manager software (Bio-Rad). Gene expression was normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) and Ubiquitin-conjugating Enzyme H5b (UBCH5B). Each experiment was performed in triplicates and the results are the mean of 3 independent experiments. Oligonucleotides used in qPCR are presented below:

ATP2C1 (gene name for SPCA1) forward 5'-GAGGCGGGTTGTGTATGCAATG-3', reverse 5'-GATATTCAGCTTTTCTGACATAGTCC-3';

SDF4 (gene name for Cab45) forward 5'-CCATGATCCAGTGCTGCATC-3'; reverse 5'-AGGAGCAGGCGGAAGCTGAT-3';

HPRT forward 5'-TAATTGGTGGAGATGATCTCTCAAC-3'; reverse 5' TGCCTGACCAAGGAAAGC 3';

UBCH5 forward 5'-TGAAGAGAATCCACAAGGAATTGA-3'; reverse 5'-CAACAGGACCTGCTGAACACTG-3'.

Calcium measurements

We measured the concentration of calcium in the Golgi apparatus by using as a probe the chimeric photoprotein Golgi-aequorin (GoAEQ) following a previously described protocol (39, 45). Cells were electroporated with GoAEQ and seeded onto glass coverslips to confluence and allowed to grow for 1 or 3 days. To produce the functional calcium-sensitive luminescent protein, aequorin was reconstituted for 40 min at 4°C in modified Krebs–Ringer buffer (KRB; 135 mM NaCl, 5 mM KCl, 1 mM K_2PO_4, 1 mM $MgSO_4$, 5.5 mM glucose, 20 mM HEPES, pH 7.4) supplemented with 5 μM mutated coelenterazine, the Calcium-ionophore ionomycin (5 μM) and 600 μM EGTA (the latter ones reduce the calcium content of the Golgi, which is necessary for measurements). After this incubation, cells were washed 3 times with KRB supplemented with 2% bovine serum albumin and 1mM EGTA (to remove calcium mobilized from intracellular compartments), followed by 3 washes with KRB buffer containing 100 μM EGTA and then transferred into perfusion chamber of a custom built aequorinometer (45). Interestingly, while this experimental procedure was suitable for non-polarized MDCK cells, the reconstitution with coelenterazine was highly inefficient in polarized MDCK cells, indicating that the Golgi apparatus of these cells is endowed with a high $[Ca^{2+}]$. Thus, to better empty the calcium from the Golgi apparatus, cells were treated 5 minutes at 37°C in calcium-free medium containing ionomycin and 2 mM EGTA before the reconstitution.

The cells were perfused with KRB containing 100 μM EGTA at 37°C. After a 30 sec baseline recording, the perfusion solution was switched to KRB supplemented with 1 mM calcium and recording continued until the light signal reached a steady-state level. At the end of each experiment, for quantification of the intra-organellar calcium levels, the cells were perfused with a hypotonic calcium-rich solution (10 mM $CaCl_2$ in H_2O) supplemented with 0.1 % TX-100 to discharge the remaining aequorin pool. The aequorin luminescence data were calibrated off-line into $[Ca^{2+}]$ values using an algorithm as previously described (45).

Immunofluorescence

MDCK cells grown on coverslips for 1 or 3 days were washed with phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$, fixed with 4% paraformaldehyde or methanol (for

SPCA1 staining) depending on the antibodies used, quenched with 50 mM NH_4Cl, and stained with specific antibodies (SPCA1 1/500 or Cab45 1/500 and TGN46 1/500 or Giantin 1/400 and furin 1/100, PLAP 1/300) in permeabilized (0.2% TX-100) conditions. Primary antibodies were detected with Alexa 488 or 546 (ThermoFisher Scientific). The images were acquired using a laser scanning confocal microscope (LSM 510 or LSM 700; Carl Zeiss MicroImaging, Inc.) equipped with a Plan Apo 63× oil immersion (NA 1.4) objective lens. The quantification of mean fluorescence intensities in random selected regions of interest was performed using the LSM 510 software (Carl Zeiss MicroImaging, Inc.) as previously described in (47). Co-localization analyses were carried out using Image J. Specifically, the Pearson's coefficient was measured using Costes' method with the Jacob plugin in Image J. The analyses were carried out on a Z-stack of images (16 bit) by considering individual cells manually defined as a region of interest.

Labelling of endogenous GPI-APs

The bacterial toxin aerolysin binds GPI-APs with high affinity. Specifically, the aerolysin mutant, ASSP, carrying a double mutation (residues 202 and 445 are changed to cysteines) that remains inactive unless it becomes reduced, was used to assess the localization of endogenous GPI-APs. CTRLi or Cab45i cells, grown on filters, were incubated with ASSP-alexa 488 (20 mM) for 1h at 4°C as previously reported (51) before fixation and confocal microscopy analysis.

N&B experiments

The Number & molecular Brightness method, a technique based on moment-analysis for the measurements of the average number of molecules and brightness in each pixel in fluorescence microscopy images (37), provides the state of aggregation of molecules in living cells with high spatial and temporal resolution. N&B experiments were carried out as previously described (1).

Microscopy and image analysis

50 frame time-series were acquired with a Zeiss LSM 510 META equipped with a plan apo 63x oil-immersion (NA 1.4) objective lens by using the following settings: 488 nm Argon laser, 0.05 mW of output power, 505-550 nm emission, gain less or equal to 850, offset 0.1, digital gain 1. Scanning parameters were: 512x512 frame window, 25.61 µs/pixel dwell time, no average, zoom 6x, ROI (x, y) 256x64, pinhole corresponding to 1 mm optical slice. Images were collected with resolutions of 70 nm/pixel. All

measurements were performed in cells displaying comparable levels of fluorescence intensity. Data from each cell were analyzed by SimFCS software (Globals Software, East Villa Grove, IL 61956, USA) following a described procedure (37). A correction was applied for taking into account the analog detection of fluorescence by the photomultiplier tubes of the confocal microscope in order to express the molecular brightness (e) in terms of photons/s/molecule (36). Briefly, the correction parameters S (the conversion factor between one photon detected and the number of digital levels produced by the electronics), offset and sigma0 were determined for each experiment by plotting the measured average intensity (<I>) vs the average variance (<Var>) of 50 frame time-series acquired using the same settings as above except that 4 different values of the laser transmission percentages and filters and beam splitters configured to get reflection images, in order to detect the defined amount of light originating directly from the laser. The obtained plots were linearly interpolated and the equation of a straight line ($R \geq 0.99$) was used to extract the parameters S and offset based on the following equation: $\langle Var \rangle = S * \langle I \rangle + q$ (parameter related to readout noise). The parameter sigma0 was estimated from time-series acquired with laser off, as the half maximum width of the histogram peak of the dark-counts. Its value was constantly lower than 0.1, and consequently was assumed to be zero in all the calculations.

In an analog system, the brightness was calculated pixel by pixel from the following equation $B= V(x,y)/(S*Ix,y)$ and the relationship with molecular brightness is described by the following equation: $B/S= e+1$. Here we indicate with the term brightness the ratio B/S. Hence, the measured brightness (B/S) is > 1 from the pixels with mobile components, while B/S= 1 from the pixels with immobile features.

Photobleaching correction (photobleaching rate measured from the experimental data) has been included in the algorithms used to analyze N&B data (37). Specifically, we used a high-pass filter to the intensity as a function of time of each pixel, which we experimentally verified to be able to remove slowly varying signals. After removal of the trend, we added a constant equal to the average intensity at that pixel. Therefore, the variance of the ‘‘immobile’’ part is unaffected by bleaching after correction and we can recover the variance of the mobile part (37).

In all experiments a detrend function (the same used for bleaching correction) was applied to image stacks before determining the B in order to avoid that slow changes of the intensity due to the cell movement or protrusion/retraction events could interfere with our measurements (36, 37). Finally, all acquisitions where we monitored aberrant movements (e.g., microvilli movement or fluctuations of the apical membrane) were discarded.

Data Analysis

As previously showed (1), by using the K-means function in Matlab (The Mathworks Inc. Natick, MA) we partitioned, with an interval of 0.5, the observed brightness values upon different experimental conditions into N exclusive groups with statistical reliability. In particular, for each experiment (number of cells > 15) we obtained the percentage of pixels in each group (calculated as an average of single cell values from an experiment). The range of B values were ascribed to monomer, dimer, trimer etc. on the basis of extrapolation of the standard curve obtained by plotting the experimental B values for monomeric, dimeric, trimeric GFP (mGFP, mGFP-mGFP, mGFP-mGFP-mGFP) vs number of units per aggregate (for detail see Supplementary Fig. 4 in ref. 1).