SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

Plasmids

The mEGFP-22 and mCerulean-22 constructs under the CMV promoter have been described (Ronchi et al., 2008). tdTomato-22 was subcloned from mCerulean-22 by replacing the mCerulean coding sequence with tdTomato (Clontech Laboratories, Inc., Mountain View, CA). VSVGAxA-EGFP was generated from VSVG-EGFP in pCDNA3 (a kind gift of J. Lippincott-Schwartz, NIH, Bethesda, MD) using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) with the following mutagenic oligonucleotides (mutations in italic bold). Forward primer: 5'GACAGATTTATACAG***C***CATAG***C***GATGAACCGACTTGGAAG 3'; Reverse primer: 5'CTTCCAAGTCGGTTCATC***G***CTATG***G***CTGTATAAATCTGTC 3'. The Cerulean-22 YTDIE construct was generated from mCerulean 22 by BspEI and BamHI mediated excision of the linker region (comprising a gly-ser spacer and the myc epitope) between mCerulean and the extended cyt b5 tail. The excised linker was replaced with a BspEI-BamHI protruding oligonucleotide cassette, coding for the diacidic sequence (YTDIE) upstream to the gly-ser linker region.

Upper strand (nucleotides coding for the YTDIE sequence are in bold): 5'CCGGAGAG CAGAAGCTTATCTCT**TACACAGACATCGAA**GGTGGAGGAGGTTCAGGAGGAGGTGGATCT GGAGGTGGCG3'

Lower strand: 5'GATCCGCCACCTCCAGATCCACCTCCTCCTGAACCTCCTCCACCTTCGA TGTCTGTGTAAGAGATAAGCTTCTGCTCT3'

Wild type and GTP-blocked (Q71L) Arf1 in the pLex vector, kind gifts of B. Goud (Institut Curie, Paris, France) were subcloned into the Sal1 and EcoR1 sites of pCDNA3. The Syb2 coding sequence in the pSPUKT vector was amplified by PCR with the following primers:

forward: 5'ACCATGTCGGCTACCGCTGC 3'

reverse: 5' GGGACTTAAGTGCTGAAGTAAACGATG 3' , and subcloned into the pCDNA3 vector.

Other plasmids (all coding sequences under the CMV promoter) used in this study were kind gifts from the indicated investigators: mRFP-KDEL, E.L. Snapp (Einstein College of Medicine, NY); GalNAcT2-mCherry (referred to here as GalNAc-mCherry), R. Pepperkok (EMBL, Heidelberg, Germany); VSVG-EGFP, J. Lippincott-Schwartz (National Institutes of Health, Bethesda, MD); EGFR-EGFP, P. De Camilli (Yale University Medical School, New Haven, CT); myc-Rab6A (referred to here as Rab6) wt and T27N, B. Goud (Institut Curie, Paris, France).

Image and data analysis

Fluorescence intensity was quantified with ImageJ software (version 1.46, NIH, USA), and statistical analysis was performed with GraphPad Prism, version 4.0a (GraphPad Software, Inc.).

For analysis of fluorescence recovery after photobleaching, whole cell fluorescence was determined at each time point, and the fluorescence intensity of the analysed ROI was corrected to the pre-bleach fluorescence of the whole cell. ROI fluorescence intensities were then normalized to the pre-bleach ROI value. To measure retrograde transport from the Golgi to the ER, inverted FRAP experiments were performed, in which the entire ER was bleached, and the rate of Golgi emptying was determined. In this case, values in the Golgi

were normalized to those registered at t0 (the first time point recorded after bleaching). In experiments, in which Golgi emptying and ER recovery were registered simultaneously (Figs 3 and 7), both the ER ROI and the Golgi fluorescence intensities were normalized to pre-bleach values, to facilitate comparison within the same graph.

First order rate constants of Golgi emptying in inverted FRAP experiments were calculated by fitting the experimental data in the time window between 20 and 30 minutes postbleaching (a period in which the diffusion of proteins within the ER has reached equilibrium) with a monoexponential decay curve approaching a plateau, in which the ratio of Golgi to ER fluorescence is assumed to return to the pre-bleach value:

$F_{Golgi}(t) = \Delta F_{Golgi} * \exp(-k*t) + plateau$

where ΔF_{Golgi} is the difference between the maximum and minimum integrated fluorescence intensities of Golgi in the investigated time window and plateau is defined by the following relation:

$(F_{Golgi\text{-}pre}/F_{whole\text{-}pre}) = (F_{Golgi\text{-}plateau}/F_{whole\text{-}plateau})$

where F_{Golgi} is the integrated fluorescence intensity of the Golgi apparatus and F_{whole} of the whole cell (corresponding to ER + Golgi) before bleaching (pre) or after equilibrium is reached (plateau). Since F_{whole} is constant after bleaching, the fluorescence signal of Golgi at plateau was determined with the following equation:

$(F_{Golgi\text{-}plateau}) = (F_{Golgi\text{-}pre}/F_{whole\text{-}pre})*(F_{whole\text{-}post})$

where $F_{whole\text{-}post}$ is the integrated fluorescence of the whole cell at time 0 (immediately after the bleach).

For analysis of the effect of H89 treatment, integrated fluorescence intensities of the Golgi were plotted over time after subtraction of the ER contribution in the Golgi region at the corresponding time points; this was estimated by the integrated fluorescence of an equal area in the immediate surrounding of the Golgi, identified by GalNAc-mCherry staining. Golgi

fluorescence was normalized to the total fluorescence of the cell to correct for potential photobleaching.

The Golgi distribution of VSVGAxA-EGFP in fixed HeLa cells transfected either with Arf1 or Rab6 constructs were measured on line scans (15 pixels width) along the major axis of the Golgi area (identified by juxtanuclear ERGIC-53, Cy3-STxB or myc-Rab6 staining); the scans included 2 μm of the flanking ER on each side. After background subtraction, the boundary between ER and Golgi was established as the midpoint of Golgi fluorescence increase on the two sides of the scan. Mean pixel intensities within the scan in the ER and Golgi region were then determined.

Surface expression of wt and mutant VSVG-EGFP was quantified on z-stacks of surface-labelled cells (see *VSVG surface labelling*). In each section, a ROI was drawn to include all the cells in the image and the mean fluorescence intensity was determined in the antibody and in the GFP channel after background subtraction. The mean values of all sections of the stack were summed, and the fluorescence ratio between the surface signal and the total EGFP signal was calculated.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Transport of VSVGs EGFP and FPs 22 through the secretory pathway. A: Schematic representation of transmembrane cargoes analysed in this study. FP-22, Cerulean-22 YTDIE and Syb-2 are TA proteins, while VSVG-EGFP, VSVGAxA-EGFP and EGFR-EGFP are type I transmembrane proteins. Fluorescent tags are always exposed to the cytosol. Mutations in the export signal of VSVG are highlighted in red. B: NRK cells were co-transfected with VSVG-EGFP and GalNAc-mCherry for 24 hours at 39.3°C and then imaged alive at 32°C. Only the GFP channel is shown. Bar, 10 μm. C: Time course of VSVG fluorescence changes in the Golgi apparatus (identified by GalNAc-mCherry staining) after

temperature shift to 32°C (*n*= 11). D: Time course of CeruleanS 22 transport along the secretory pathway (see Supplementary Movie S1). NRK cells were microinjected with cDNAs specifying CeruleanS 22 and GalNAcS mCherry (not shown) and incubated for 60 min at 37°C before liveS cell imaging at the same temperature. Bar, 20 μm. E: Time course of CeruleanS 22 fluorescence variations in the Golgi apparatus (identified as GalNAcS positive region) at 37°C (*n* = 5 cells). Bar, 30 μm.

Figure S2. Characterization of VSVGAxA and CeruleanS 22 YTDIE constructs. A: Transport of VSVGAxA to the plasma membrane. NRK cells were transfected for 24 hours at 39°C and then incubated at 32°C for the indicated times before fixation. Arrowheads indicate the weak enrichment of mutant VSVG in the Golgi apparatus (identified by GalNAc-mCherry staining), while the arrows highlight the progressive arrival of the protein at the plasma membrane starting at 2 h of incubation at 32°C. Bar, 15 μm. B: VSVGAxA is properly folded after incubation at 20°C. NRK cells were transfected for 24 hours at the non-permissive temperature and either fixed or incubated for further 90 min at 20°C before fixation. Cells were then processed for immunofluorescence with the conformation-specific antiS VSVG antibody (clone IE9F)). Single optical sections were captured in both EGFP and antibody channels using the same acquisition settings for all images. Bars, 15 μm. C: CeruleanS 22 and CeruleanS 22 YTDIE are both expressed at the plasma membrane in NRK cells after transfection at 37°C for 24 h. Bar, 20 μm.

Figure S3. VSVGAxAs EGFP fails to acquire EndoHs resistance at 20°C.
HeLa cells were transfected with VSVGS EGFP (upper) or its signalS deficient mutant (lower) for 24 hours at 39.3°C, and incubated at 20°C for the indicated times before lysis. Lysates were incubated

with or without EndoH and analysed by SDS-PAGE-immunoblot with anti-GFP antibodies. EndoH-R and -S indicate the positions of the EndoH–resistant and sensitive VSVG forms.

Figure S4. Lack of evidence for interaction of VSVG with adaptin complexes within the Golgi complex at 20°C. A: Silver stained gel of VSVG immunoprecipitates. Lysates were prepared from non-transfected cells or from cells transfected with VSVG-EGFP. Cells were kept at 39°C for 24 h, followed by incubation at 20°C for 2 h. Immunoprecipitation was carried out with anti-GFP or non-specific IgG, as indicated. The arrow indicates the band corresponding to VSVG-GFP, while the arrowhead indicates a band present only in anti-GFP immunoprecipitates obtained from transfected cells. H_c and L_c indicate the heavy and light Ig chains. B: Immunoprecipitates, obtained as in (A), were analysed by western blotting with anti-GFP and anti-γ and δ adaptin antibodies. C: The distributions of VSVG-EGFP and AP1 or AP3 were compared after expression of VSVG-EGFP in HeLa cells for 24 hours at 39.3°C followed by incubation for 90 min at 20°C. Cells were fixed and immunostained with - anti-AP1 δ-chain or anti-AP3 γ chain mAbs, as indicated. The boxed areas are enlarged in the insets; the graphs show the line scan of VSVG and adaptins along the yellow arrows in the Merge panel. Only a partial colocalisation is observed Bars, 10 and 4 μm (insets).

Figure S5. Golgi tos ER retrograde trafficking of VSVGAxAs EGFP is inhibited in cells overexpressing dominant negative Rab6. HeLa cells were coS transfected with VSVGAxAS EGFP and GalNAcS mCherry (CTRL: A) or with VSVGAxAS EGFP and Rab6 T27N (B) for 24 hours at 39.3°C; after incubation for 20 min at 4°C in the absence (A) or presence (B) of Cy3S STxB, followed by a further 5 hours at 32°C, the cells were live imaged at 20°C. The red contour indicates the bleached area. Bars, 10 μm.

LEGENDS FOR VIDEOS

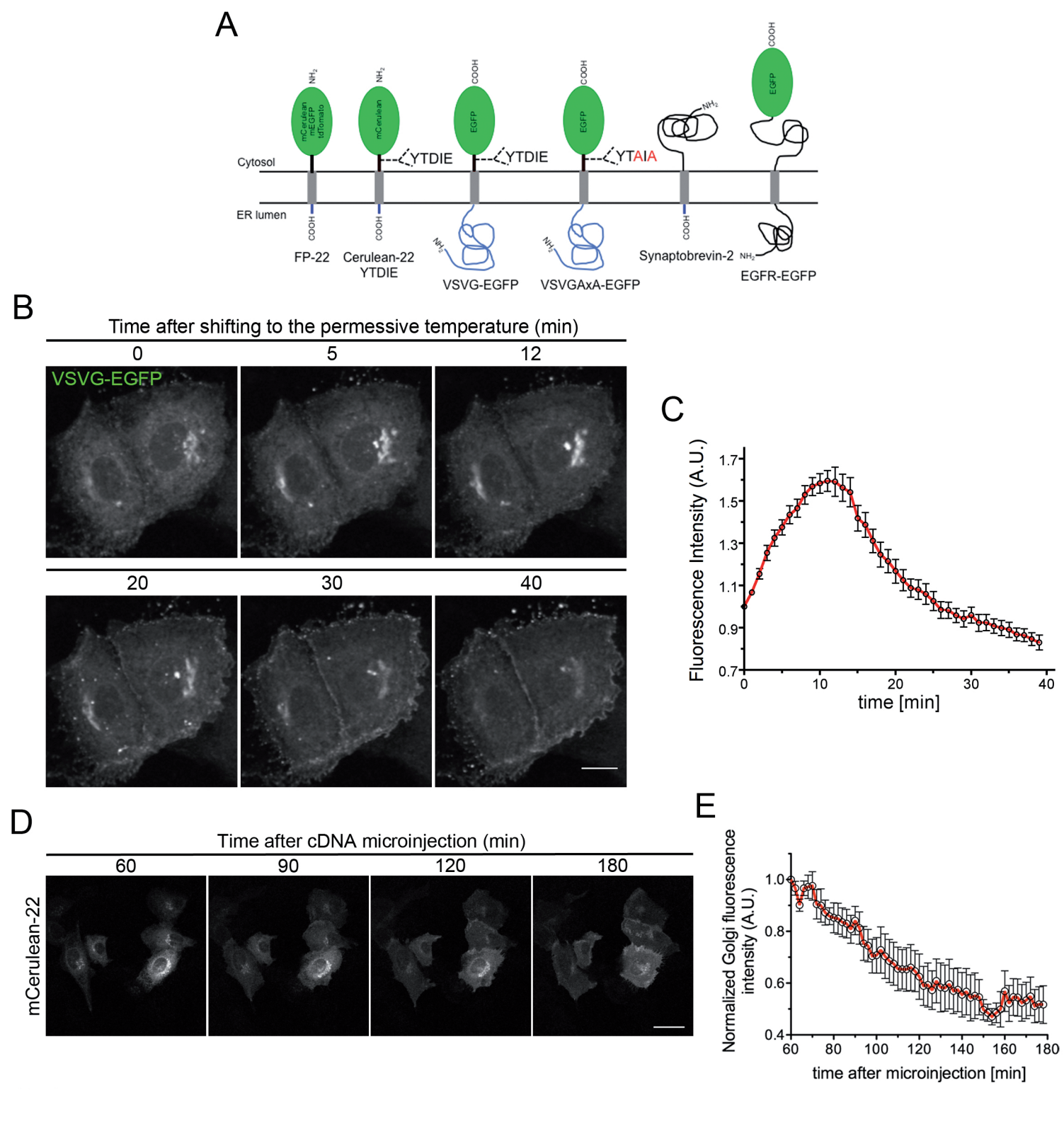
Movie S1. Time course of FPs 22 transport through the secretory pathway at 37°C (related to Fig. S1). The distribution of CeruleanS 22 progressively changes over time from an ER/Golgi localization to a typical plasma membrane distribution (see legend to Fig. S1 for details). Note that the appearance of the plasma membrane is accompanied by a progressive emptying of both the ER and the Golgi apparatus.

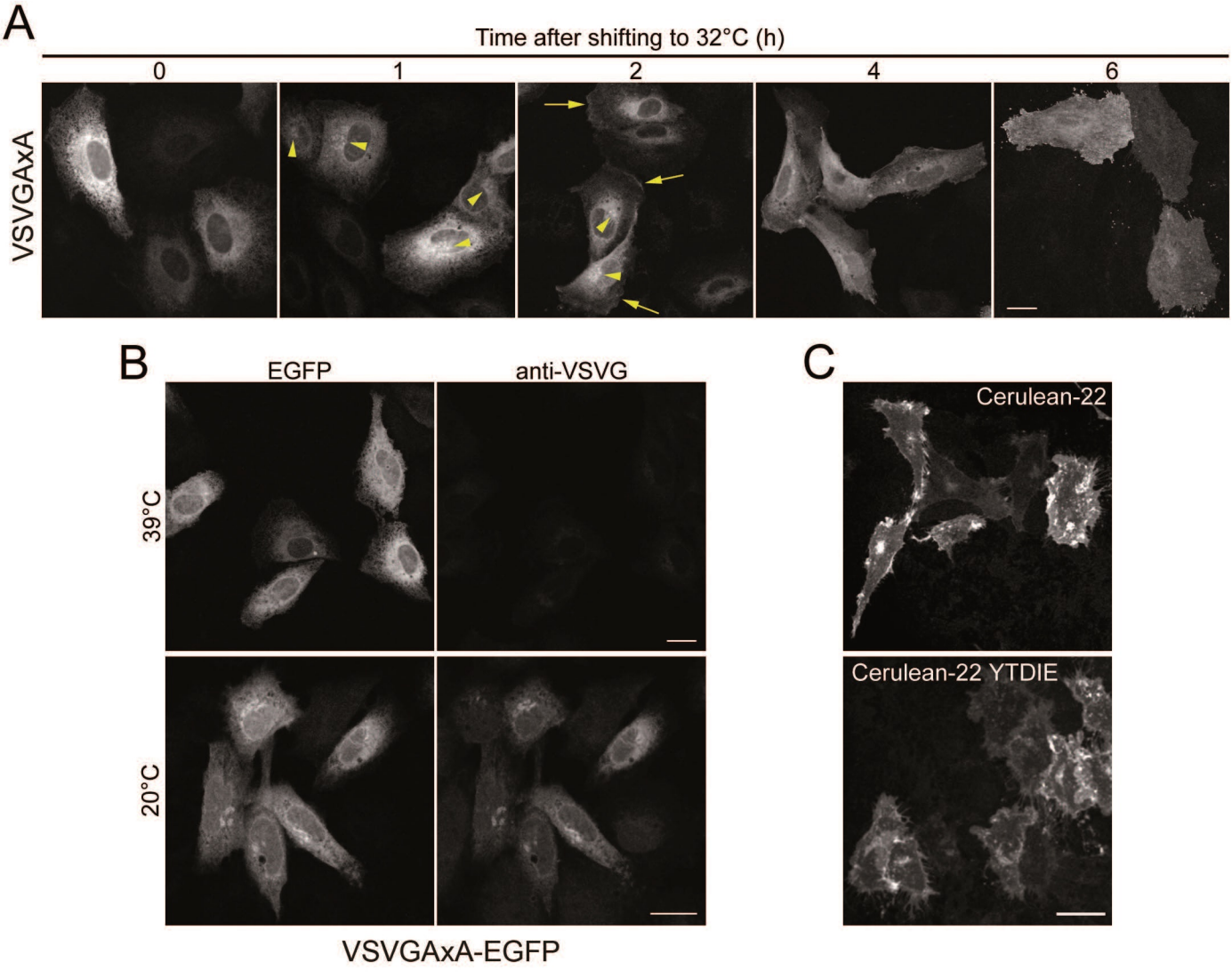
Movie S2. Anterograde transport of FPs 22 from the ER to the Golgi at 20°C (related to Fig. 1). Photobleaching of Golgi apparatus (red contours) and of a portion of ER (blue contour) at 20°C (see legend to Fig. 2 for details). Note an FP-22-positive vesicle in the cell located in the lower right corner of the field that leaves the ER and fuses with the Golgi apparatus (highlighted by the red arrowhead).

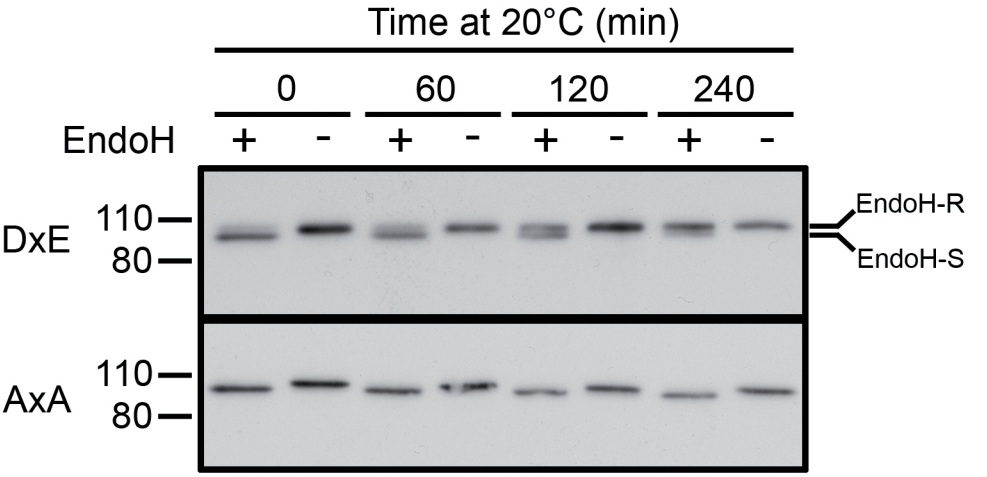
Movie S3. ERs tos Golgi anterograde transport of FPs 22 is inhibited in energys depleted cells (related to Fig. 1). Representative FRAP experiment of Golgi apparatus (red contour) in energyS depleted NRK cells (see legend to Fig. 1 for details).

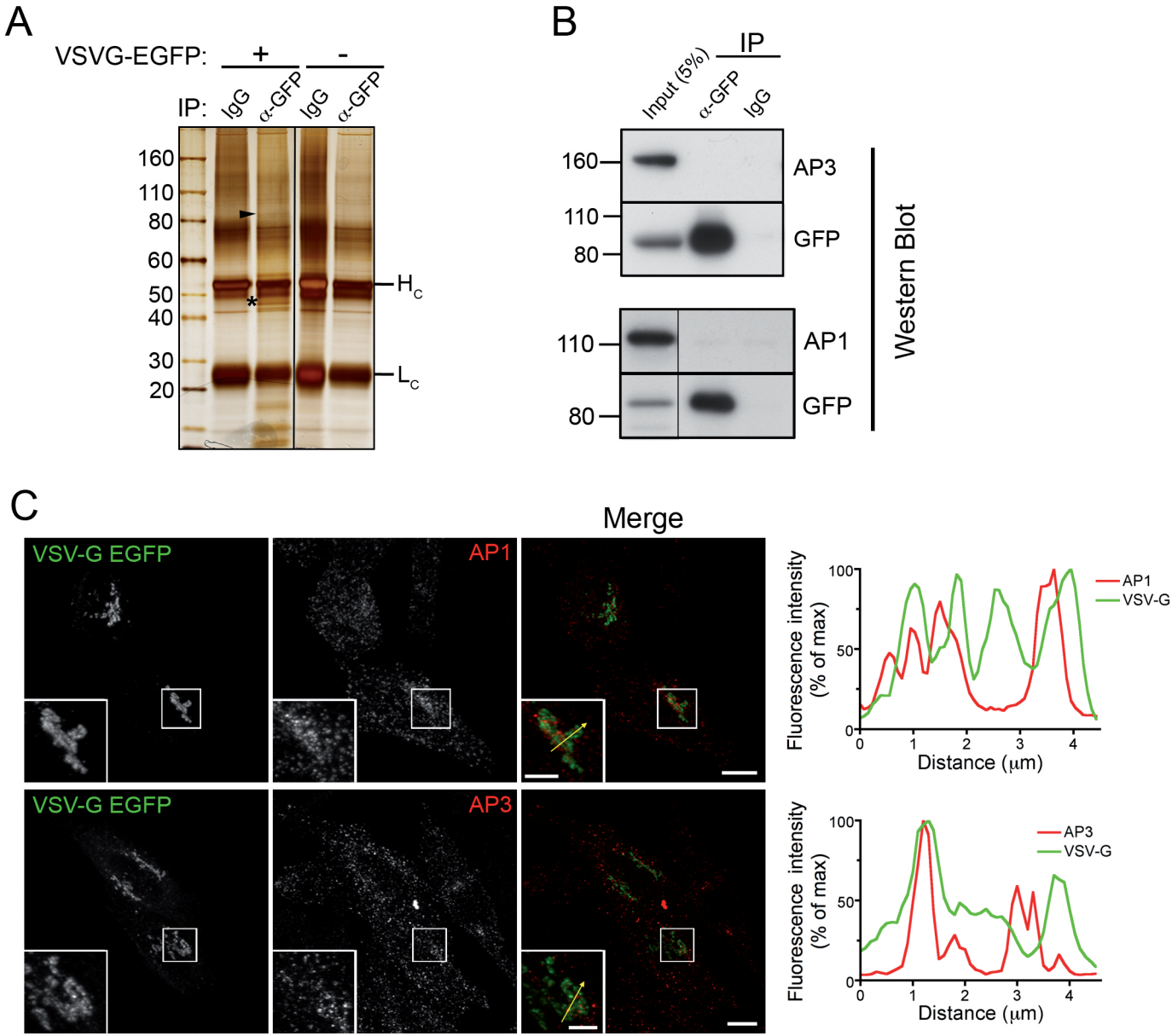
Movie S4. FPs 22 is included into a Golgis tos ER retrograde transport pathway (related to Fig. 2). Photobleaching of FPS 22 ER (red contour) at 20°C (see legend to Fig. 2 for details). Note that after ER bleaching, in particular in the cell on the left, many tubular/ vesicular structures positive for FPS 22 emerge from the Golgi region and disperse into the cell periphery.

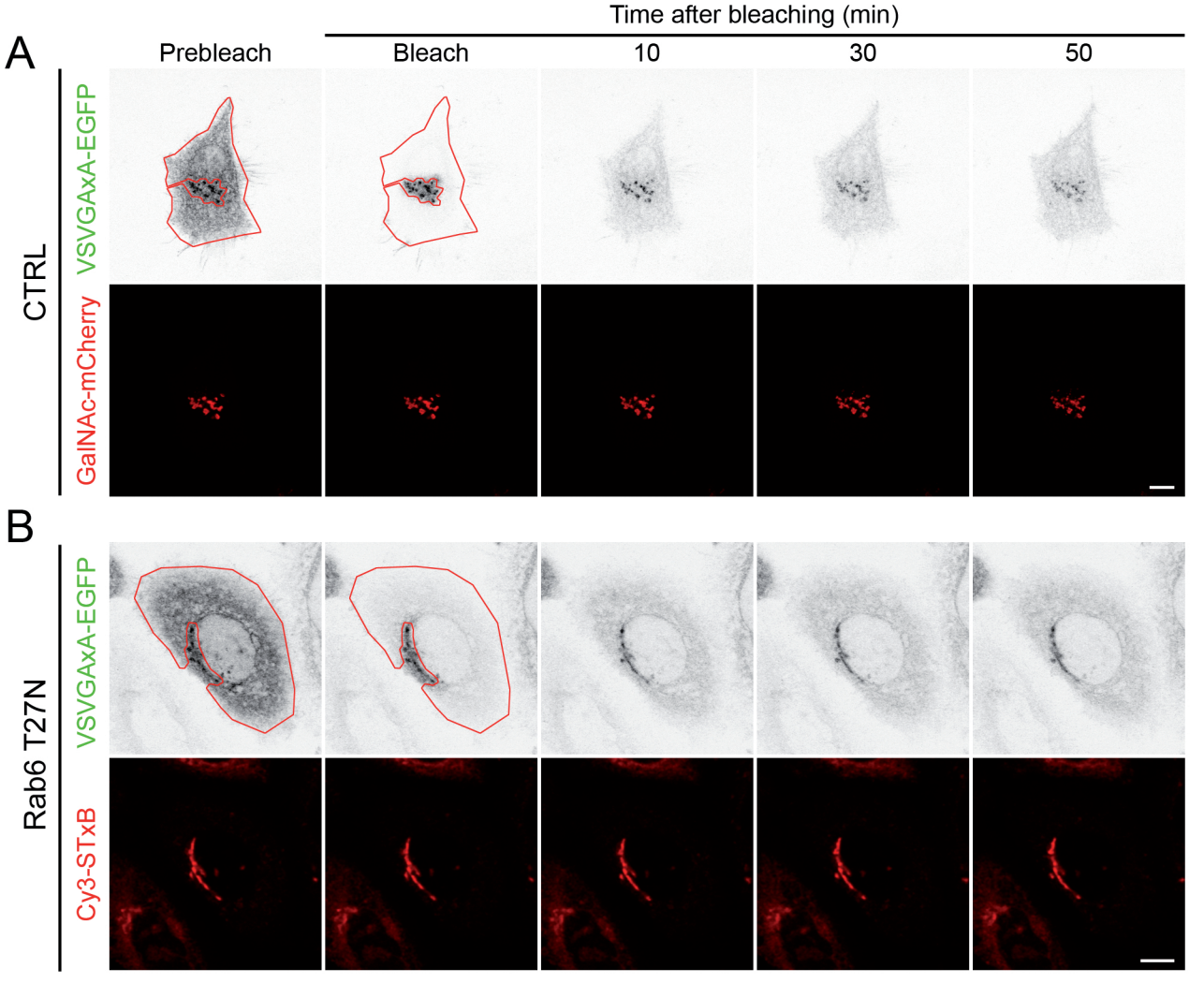
Movie S5. The signals deleted mutant of VSVG is recycled from the Golgi to the ER (related to Fig. 6). Photobleaching of the ER (red contour) in VSVGAxAS EGFP overexpressing cells at 20°C (see legend to Fig. 6 for details).


A
mCerulean
mEGFP
tdTomato
mCerulean
EGFP
EGFP
EGFP
Cytosol
ER lumen
YTDIE
YTDIE
YTAIA
FP-22
Cerulean-22
YTDIE
VSVG-EGFP
VSVGAxA-EGFP
Synaptobrevin-2
EGFR-EGFP
B
Time after shifting to the permessive temperature (min)
0
5
12
VSVG-EGFP
20
30
40
C
Fluorescence Intensity (A.U.)
time [min]
D
Time after cDNA microinjection (min)
60
90
120
180
mCerulean-22
E
Normalized Golgi fluorescence intensity (A.U.)
time after microinjection [min]


A
Time after shifting to 32°C (h)
0
1
2
4
6
VSVGAxA
B
EGFP
anti-VSVG
39°C
20°C
VSVGAxA-EGFP
C
Cerulean-22
Cerulean-22 YTDIE


Time at 20°C (min)
0
60
120
240
EndoH
+ - + - + - + -
DxE
110
80
AxA
110
80
EndoH-R
EndoH-S


A
VSVG-EGFP: + -
IP: IgG α-GFP IgG α-GFP
160
110
80
60
50
40
30
20
H_C
L_C
B
IP
Input (5%)
α-GFP
IgG
160
AP3
110
GFP
80
110
AP1
80
GFP
Western Blot
C
Merge
VSV-G EGFP
AP1
VSV-G EGFP
AP3
Fluorescence intensity (% of max)
100
50
0
0 1 2 3 4
Distance (μm)
AP1
VSV-G
Fluorescence intensity (% of max)
100
50
0
0 1 2 3 4
Distance (μm)
AP3
VSV-G


A
Time after bleaching (min)
Prebleach
Bleach
10
30
50
CTRL
VSVGAxA-EGFP
GalNAc-mCherry
B
Rab6 T27N
VSVGAxA-EGFP
Cy3-STxB