Supplementary data

Materials and Methods

Antibodies

The following primary antibodies were used in this study: polyclonal antibody against HA (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:1000; anti-myc antibody (Clone 9E10, Santa Cruz) 1:500; anti-FLAG antibody (M2, Sigma, St. Louis, Missouri) diluted 1:800; anti-Xpress antibody (Invitrogen Corporation, Carlsbad, CA) diluted 1:200, affinity-purified rabbit polyclonal antibody raised against a synthetic peptide corresponding to the carboxy-terminal residues of human LIMK1 (Cell Signaling Technology, Danvers, MA) 1:1000; rabbit polyclonal raised against amino acids 136-219 mapping near the N-terminus of human LIMK1 (Santa Cruz)1:100; rabbit polyclonal antibody against amino acids 561-638 mapping at the C-terminus of human LIMK 2 (Santa Cruz) 1:500; rabbit polyclonal antibody against phosphorylated cofilin (provided by J. Bamburg, Colorado State University, Fort Collins, CO); (Meberg and Bamburg, 2000) diluted 1:1000; rabbit polyclonal antibody against nonphosphorylated cofilin (Sigma) 1:5000; actin (C-2) antibody (Santa Cruz) 1:1000; antibody against the ectodomain of p75 (clone ME 20.4; hybridoma provided by M. Chao, New York University), diluted 1:500 from ascites fluid; monoclonal antibody against dynamin 2 (HUDY-1; 1:200 dilution of ascitic fluid; provided by S. Schmid (Warnock *et al.*, 1995); a rabbit antibody against TGN 38 (Cao *et al.*, 2000), was used at a 1:200 dilution for TGN staining; and the antibody that specifically recognize the carboxyl terminus tyrosine residues (C-Tyr) of cortactin (Cao et al., 2005), (provided by M. McNiven).

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Plasmids

The expression plasmids used in this study encoded the following proteins: (1) GFP tagged-p75 neurotrophin-receptor (p75-GFP) (Kreitzer *et al.*, 2000); (2) GFP-tagged-chicken neuronal cell adhesion molecule (NCAM-180) fused to the N-terminus of enhanced GFP (NCAM-GFP) (Kreitzer *et al.*, 2003); (3) Hemagglutinin (HA)-tagged LIMK1-KD, and (4) FLAG tagged-constitutively active cofilin (Cofilin S3A) (Sumi *et al.*, 1999; Rosso *et al.*, 2004). 5) Kinase dead LIMK2 (LIMK2-KD) was a generous gift of James Bamburg, Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO. Other expression plasmids included:

(6) ST-mRFP, the N-terminal 37 amino acids of sialyltransferase encoding a TGN retention signal was amplified by the polymerase chain reaction (PCR) using the following primers 5‘-GCGAATTCATGATTCACACCAACCTG-3’and 5’-TACGGAT CCGAATCATAGT-AACTCCC-3’. The resulting product containing an EcoRI site upstream of the initiation codon and a BamHI site in frame with the mRFP was ligated into the EcoRI and BamHI sites of the mRFP-N1 vector (based on pEGFP-N1 from Clontech), resulting in ST–mRFP. Stable transformants were selected in 500 μg/ml G418 (Gibco Laboratories). G418-resistant clones were isolated with cloning rings and screened for ST-mRFP expression; (7) Galactosyltransferase-CFP (GalT-CFP). Stable transfectants of MDCK cells were obtained by transfection with GalT-CFP (provided by Jamie White, Massachusetts General Hospital Cancer Research Center, Charlestown, Massachusetts, USA). Selection of transformants was performed by L. Leung; (8) Wild-type dynamin-2bb cDNA (Kreitzer *et al.*, 2000); (9) Xpress-Syndapin 2 SH3; (10)

Syndapin 2-GFP; (11) myc-tagged Dynamin 2 aa ΔPRD (dyn2ΔPRD) provided by B. Qualmann (12) DsRed-Cortactin B lacking 50 amino acids of the SH3 domain (CortΔSH3). (13) myc-tagged Dynamin 2 PRD domain (Dyn 2-PRD). These plasmids were provided by M. McNiven (Mayo Clinic, Rochester, MN, USA), (14) Actin-Photoactivatable GFP (actin-paGFP). PaGFP cDNA (J. Lippincott-Schwartz, Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health) was amplified by PCR using 5'-TGGAAGCTTCCACCATGGTGAGCAAGGGC-3' and 5'-CCGGATCCCTTGTACAGCTCGTCCATGC-3'. The resulting product containing a Hind III site upstream of the initiation codon and a BamHI site in frame with the paGFP was ligated into the HindIII and BamHI sites of the pcDNA-EGFP-beta-actin vector (B. Imhof, Department of Pathology, Centre Medical Universitaire, Geneva, Switzerland), resulting in actin-paGFP. Stable MDCK II clones expressing actin-paGFP were isolated with cloning rings and selected with 1 mg/ml G418. 15) NHR2-GFP provided by Moses V. Chao (New York University School of Medicine, New York, USA) and 16) GPI-YFP was a generous gift of Dr. M Lippincott-Schwartz (National Institutes of Health, Bethesda, MD 20892, USA).

Immunofluorescence

Cells were fixed in 4% PFA in PBS for 10 min, followed by permeabilization in PBS containing 0,2% Triton X-100. Primary antibodies were diluted in PBS with 1% BSA (bovine serum albumin). Secondary antibodies were FITC, Cy3 and Cy5-conjugated conjugated all from Jackson Immunoresearch. Images were collected and stored as described for time-lapse experiments.

ER to Golgi transport

Native human p75 ^{NTR} was expressed in MDCK cells using a replication defective recombinant adenovirus vector (kindly provided by S.-O. Yoon, M. Chao, and E. Falck-Pedersen, New York University) 30 h prior to transient transfection of LIMK1-KD plasmid by nucleofection with Amaxa^{TM} technology using 20µg of cDNA per $4x10^6$ cells. Cells were analyzed 48 h after transfection. Transfection efficiency of each experiment was evaluated by immunofluorescence.

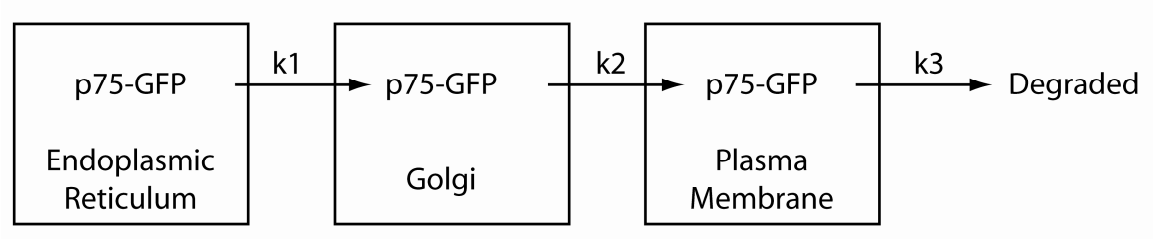
Pulse-chase analysis of the kinetics of the acquisition of endoglycosidase H resistance by p75 was carried out in MDCK cells co-expressing p75-GFP and LIMK1-KD. Briefly, cells were pulse-labeled for 20 min in [35S]methionine/ cysteine (1 mCi/ml in starving medium containing 1/10th the concentration of methionine/cysteine of regular medium; Perkin Elmer-Cetus) and chased for various times in medium containing (DMEM, 2.5 mM Met/Cys and 10% FBS) at 37°C. At each chase time, dishes were placed on an ice bath and lysed. Immunoprecipitations of human p75 was performed with a mouse mAb (ME 20.4, diluted 1:300), and immune complexes were collected by incubation for 2 h with protein A–Sepharose (Pierce). Half of the sample's volume was treated with 3 mU Endoglycosidase H at 37°C for 16h. The samples were analyzed by 12% SDS–PAGE. ^{35}S-labeled p75 was detected using uncoated phosphor-screens on a STORM 860 Phosphorimager (Amersham, Piscataway, NJ). Band intensity was quantified by Imagequant version 1.2 (Amersham). The endoglycosidase H resistant fraction of p75 was expressed as a fraction of total p75 to determine the fraction processed by Endo H at each chase time.

Statistical analysis

Data analysis was performed using GraphPad Prism, GraphPad Software, San Diego California USA. The significance of differences in the mean between samples was determined by two-paired *t*-test analysis.

Mathematical Model for Golgi exit.

Transport of proteins from the ER, to the Golgi and the plasma membrane were modeled with a series of coupled differential equations using Gepasi (Mendes, 1993) as shown in Figure 1C. Rates were determined by global optimization of the parameters to best fit the measured Golgi fluorescence data. Initial concentrations for p75-GFP_{total} and p75-GFP_{Golgi} were obtained from fluorescence at time 0 in each movie. Initial concentration of ER p75-GFP was estimated as p75-GFP_{total} - p75-GFP_{Golgi} (neglecting the presence of p75-GFP at the plasma membrane at time 0). In each movie analyzed, several optimization algorithms implemented in Gepasi were used to try and reach a global minimum. Fitting plots were inspected for each cell to verify that the procedure produced a reasonable fit. The rates corresponding to the best fit were kept and averaged over 3 movies to obtain the rates and standard deviation reported in Figure 1D.



Supplementary Figure 1: LIMK 1-KD does not inhibit ER-Golgi transport.

A) Pulse-chase analysis of the kinetics of the acquisition of endoglycosidase H resistance by p75 in control and LIMK1-KD-expressing- MDCK cells 48h after transfection. **B)** For the lanes shown in A, the endoglycosidase H resistance was normalized to total p75 to determine the % of p75 processed. Results are from two independent experiments.

Supplementary Figure 2: Overexpressed LIMK1-KD but not LIMK2-KD localizes to the Golgi apparatus.

MDCK cells stably expressing galactosyl-transferase-CFP (Gal-T, top) or Sialyltransferase-mRFP (Sialyl-T, bottom) were injected with plasmids encoding for HA-LIMK1-KD or LIMK2-KD and labeled with anti-HA antibodies 1.5 h thereafter. Note that LIMK1-KD colocalizes with Gal-T whereas LIMK2-KD labeling is throughout the cytoplasm and does not colocalize with Sialyl-T. Scale bars represent 10 μm.

Supplementary Figure 3: Overexpression of cortactin Δ SH3 **does not** inhibited exit of N-CAM from the TGN.

Supplementary Figure 4: Effect of Jasplakinolide and Cytochalasin D

A) Endogenous actin: Actin (green) was detected with phalloidin (a,b,d,e) or by immunofluorescence with anti-actin antibodies (c,f) whereas the TGN (red) was identified by expression of ST-mRFP (a,b,d,e) or by immunofluorescence with antibodies to TGN 38 (c,f). Control cells (a,b) displayed stress fibers at the cell periphery and a delicate actin filaments in the perinuclear region, in close association with the TGN; b

represents the boxed area in a. Cells treated with cytochalasin D, LIMK 1-KD, Cofilin S3A or siRNA against LIMK 1, displayed small F-actin aggregates in the cytoplasm and the Golgi region. Scale bars: 20 μm (a) or 5 μm (b-f).

B) Cells expressing p75-GFP and ST-mRFP. Representative images taken from movies show ST-mRFP. Note very long tubules containing ST-mRFP in cells treated with cytochalasin D (arrows) for 2 hours after release of the 20°C block (right). Tubules in control cells (arrows) are much shorter. Scale bars: 5 μm.

C) Overexpressed GFP-actin: GFP-actin (green) is efficiently incorporated into short and long actin filaments that are equally susceptible to the effects of the actin toxins as endogenous actin filaments. Sialyl transferase-mRFP: Golgi resident protein (red). In Jasplakinolide treated cells, actin aggregated into large clumps and some stress fibers persist. Scale bars: 20 μm.

Supplementary Figure 5: Co-expression of three proteins using triple microinjection protocol

A) cDNAs encoding p75-GFP (a), the Golgi resident protein Sialyltranferase (ST-mRFP) (b) and LIMK 1 KD (c) were co-injected and processed for inmunostaining after 1.5 h. Color overlay of Golgi (red), p75-GFP (green) and LIMK 1 KD (blue) is show in (d). Note that few cells do not co-express LIMK 1 KD (asterisks in c). **B)** cDNAs encoding p75-GFP (a), ST-mRFP (b) and Cofilin-S3A (c) were co-injected and processed for inmunostaining after 1.5 h. Color overlay of Golgi (red), p75-GFP (green) and Cofilin-S3A (blue) is show in (d). Scale bars: 20μm.

Supplementary movie 1: Dynamic behavior of p75-GFP in the TGN of control MDCK cells. P75-GFP was accumulated in the TGN after nuclear injection of its cDNA and incubation at 20^{o}C for 3 h. After release of the temperature block, p75-GFP tubules (green) are observed emerging from the TGN, recognized by the presence of ST-mRFP (red).

Supplementary movies 2 and 3: Static behavior of p75-GFP in the TGN of LIMK 1-KD and Cofilin S3A expressing cells. P75-GFP was accumulated in the TGN after nuclear injection of its cDNA and incubation at 20^{o}C for 3 h. After release of the temperature block in the presence of overexpressed LIMK1 –KD and Cofilin S3A segregation of cargo p75-GFP from TGN resident protein ST-mRFP is drastically disrupted. Cells displayed many poorly dynamic tubules containing either ST-mRFP (red) alone or ST-mRFP plus p75-GFP (green).

Supplementary movie 4: Post-Golgi carriers of a control MDCK cell expressing p75GFP. 10 min after release from the temperature block, images were collected at 1-2s intervals for 1 min. P75-GFP is seen in a perinuclear compartment and in post-Golgi transporters displacing along linear paths within the cytoplasm with typical kinesin motors speeds. Note a representative track (blue) of a transporter (arrow). The starting point of the track is shown in green and the final point in red.

Supplementary movie 5: LIMK1-KD expression reduced the number of p75-GFP containing post-Golgi carriers 10 minutes after release from 20°C. Note the relative absence of p75-GFP transporters compared with the control cell (movie 4).

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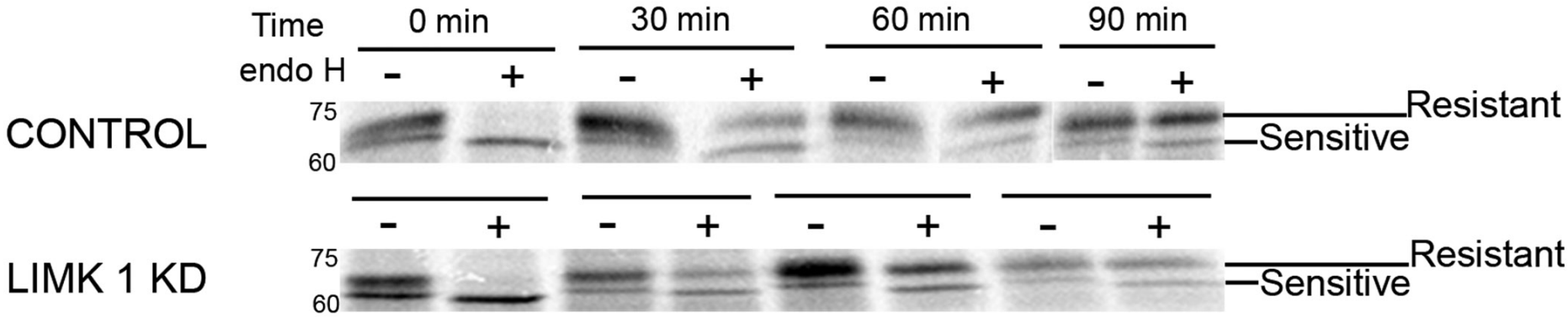
A



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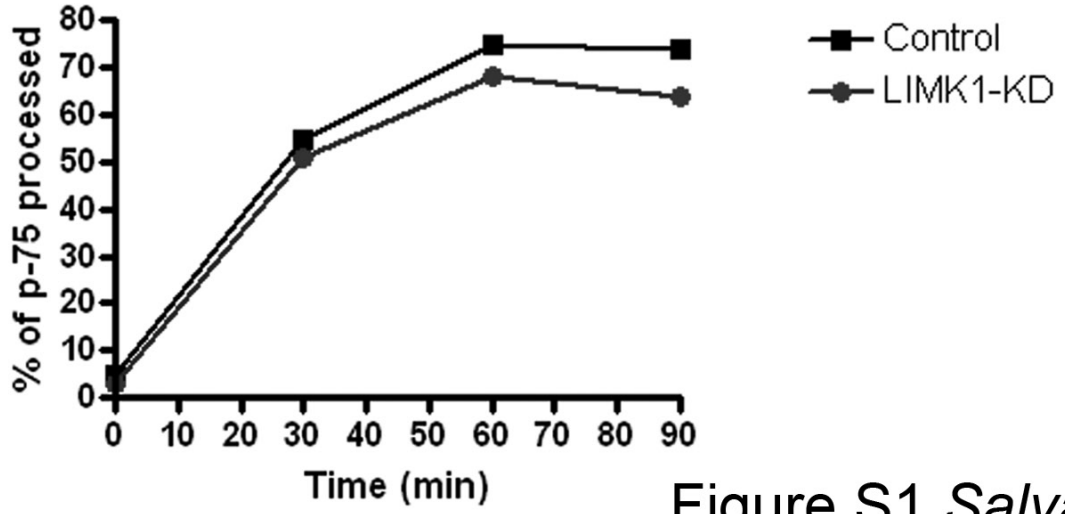

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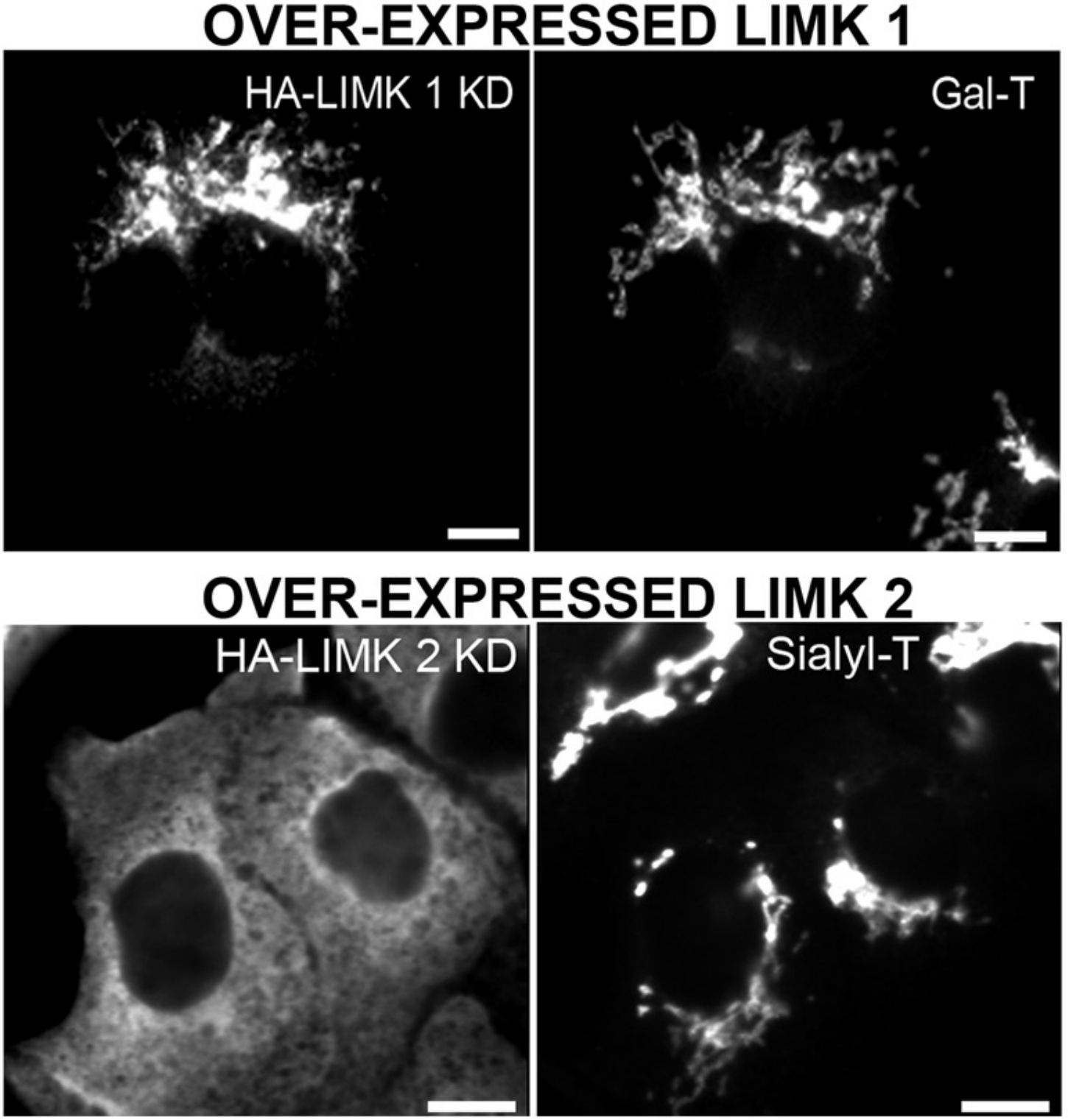

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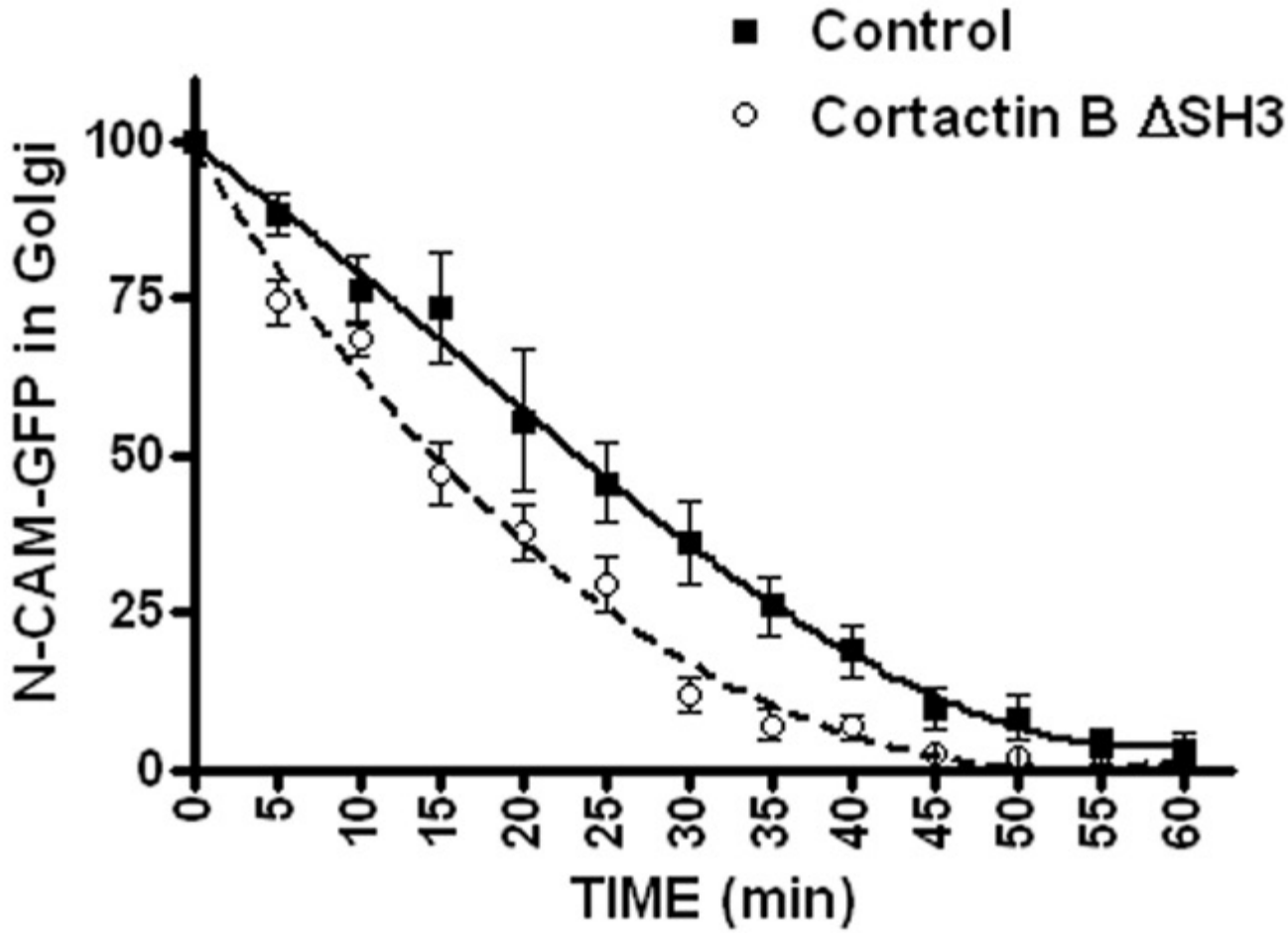

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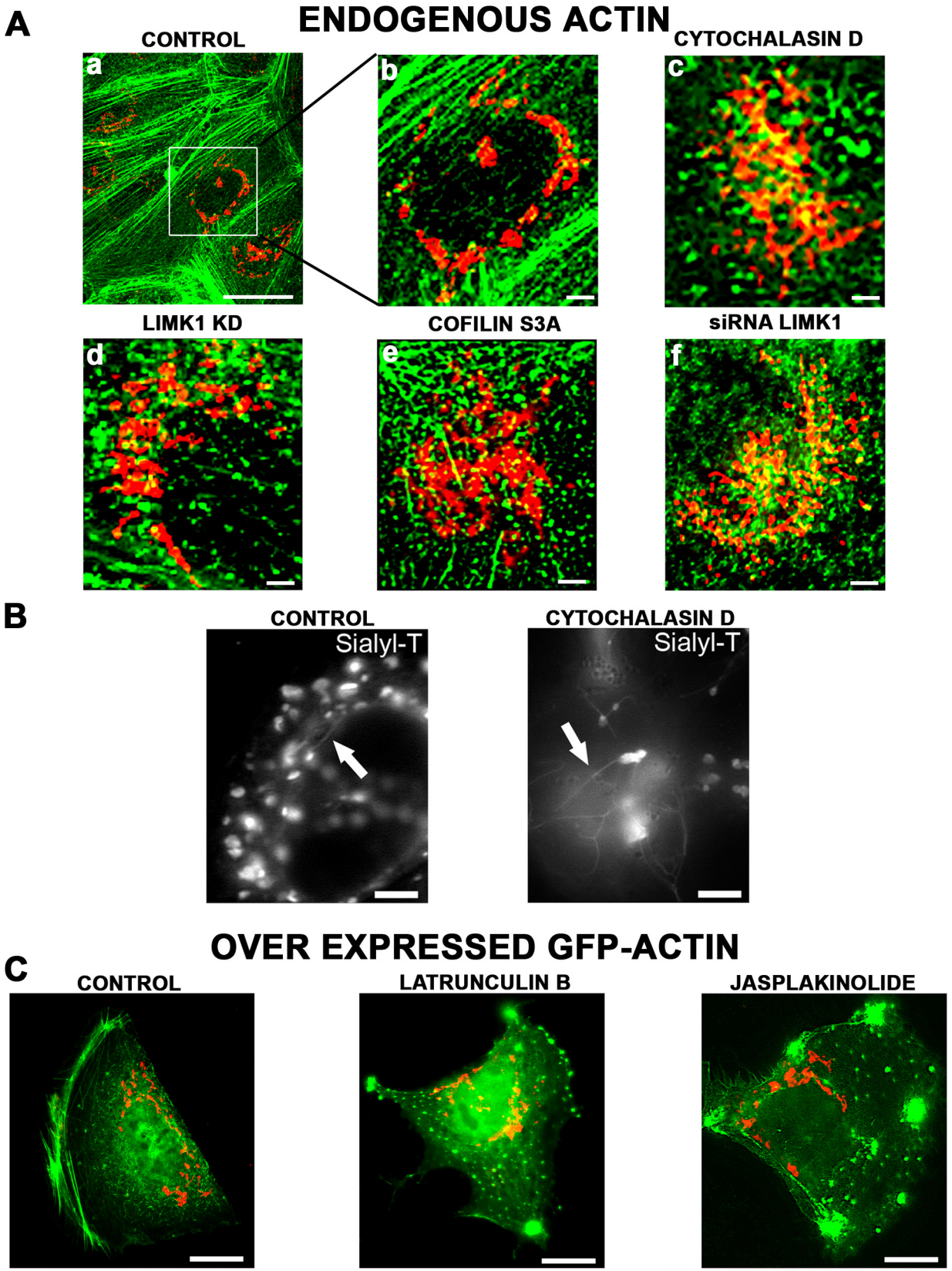

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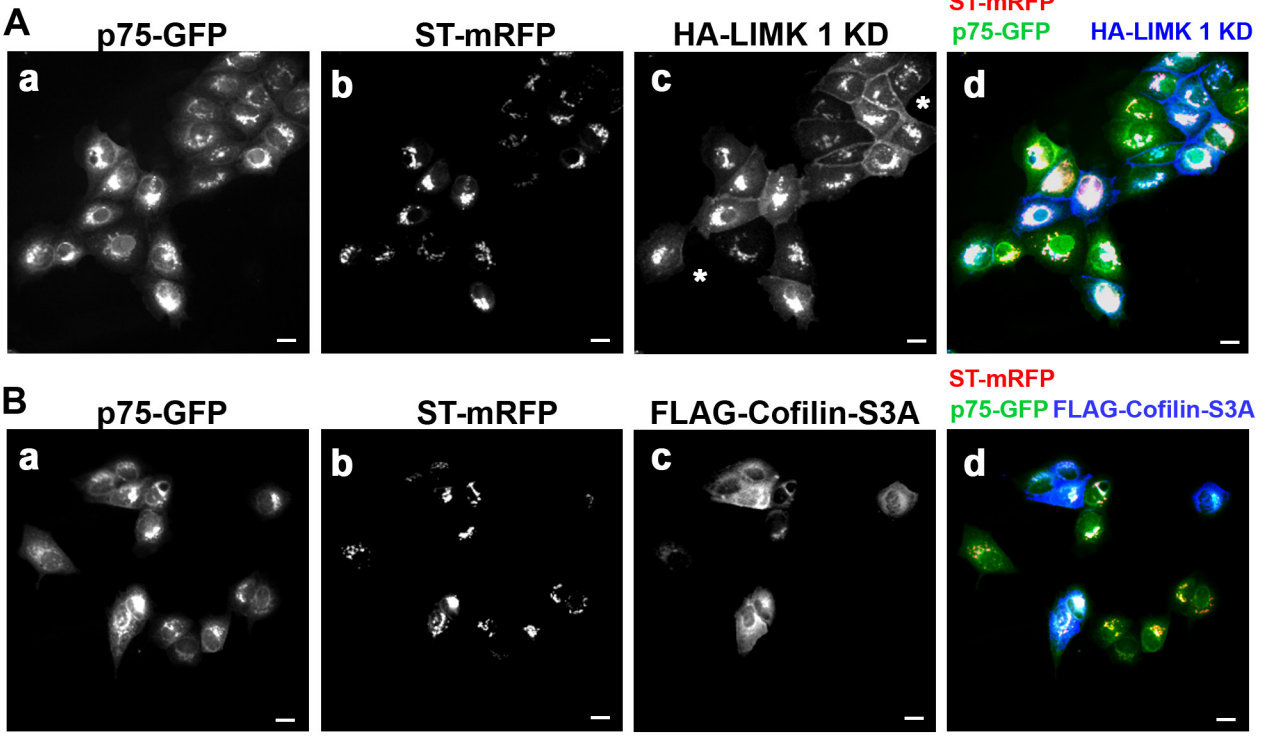

Figure S5 *Salvarezza et al.*