Supplemental materials and methods

Antibodies and reagents

Monoclonal antibodies: against calnexin, cathepsin D, and EEA1 were purchased from BD biosciences; against TfR from Zymed; and against β-actin, Myc, FLAG, and α-tubulin from Sigma; against Lamp-1 from Stressgen; against M6PR from Affinity Bioreagents; against ERGIC-53 from Alexis. Polyclonal antibodies: against Rab6 and clathrin heavy chain were purchased from Santa Cruz; against TGN46 from AbD Serotec; against GST from GE Healthcare. Polyclonal antibodies against Sec31 and β-COP were generous gifts from Dr. M. Tagaya. H89 was purchased from Millipore. PKI, blebbistatin, and latrunculin A were from Calbiochem. The plasmids encoding Rab6a-GFP, Rab8a-GFP, and Rab11a-GFP were generous gifts from Dr. F. A. Barr. The plasmids encoding PAUF-MycHis, GFP-myosin IIa, and mRFP were generous gifts from Drs. S. S. Koh, R. S. Adelstein, and M. Tagaya, respectively.

Cell culture and transfection

HeLa-ssHRP cells (a gift from Dr. I. Stamenkovic) and HeLa cells were grown in DMEM supplemented with 10% fetal calf serum. Plasmid transfection was carried out using FuGENE6 (Roche) or TransIT HeLa monster transfection kit (Mirus). Transfection of siRNA was carried out using HiPerFect transfection reagent (QIAGEN) according to the manufacturer's protocol.

RT-PCR

RT-PCR was performed using Superscript III one-step RT-PCR system with platinum Taq DNA polymerase (Invitrogen) according to the manufacturer's protocol. Each cDNA was amplified from RNA prepared from HeLa cells with the following primers.

PAUF (378 bp): 5'-GGAGGAGGCAAGTATTTCAGC-3' and 5'-ATTCCATTCAAAGCCAATGC-3'. PAUF (401 bp): 5'-GGGCAGGGAAGATGTATGG-3' and 5'-ATTCCATTCAAAGCCAATGC-3'. Syt II (486 bp) 5'-AGAATGCCATGAACATGAAGG-3' and 5'-CTTGCAGGTCTCTCCACTCC-3'. Syt II (499 bp) 5'-TTGAGGAGTGGAGAGACCTG-3' and 5'-CTACTTGTTCTTGCCCAGGAG-3'. Syt II (227 bp) 5'-CAGTGGGACTCCATGGTTCT -3' and 5'-TGTCAGACTGCAGGAGGATG -3'.

Plasmid construction

The cDNA encoding full-length human lysozyme C or Syt II was inserted into a pcDNA3-based plasmid encoding 3xFLAG to express protein with a C-terminal 3xFLAG tag. The cDNA encoding human PAUF was inserted into a pcDNA3-based plasmid encoding mRFP to express PAUF with C-terminal mRFP.

Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde at room temperature for 20 min. For clathrin heavy chain staining, cells were fixed with methanol at -20°C for 5 min. The cells were labeled with indicated primary antibodies and secondary antibodies conjugated to Alexa Fluor 488, 594, and/or 647. The samples were analyzed with a Leica SPE confocal microscope with the 63× ACS Apo NA 1.3 objective and LAS-AF software. An appropriate optical shift correction was performed with Huygens software (Scientific Volume Imaging). Image processing and quantification of transport carriers were performed with ImageJ software.

Live cell imaging

HeLa cells were transfected with a plasmid for PAUF-mRFP. Twenty hours later medium was replaced with phenol red-free Opti-MEM and cells were maintained in 5% CO_2 at 37°C during live cell imaging. Images were acquired continuously with time intervals between frames of 440 ms for approximately 3 min by use of a Leica SP5 II confocal microscope with 100× Plan Apo NA 1.4 objective and LAS-AF software. The images were processed with ImageJ software.

RNA interference

SiRNA oligos were purchased from Invitrogen. The sequence of control siRNA is 5'-CCACUUUAACUUAGACUACGCAAUU-3'. The sequences of siRNA oligos targeting human myosin IIa are 5'-CAACCUCAAGGAGCGUUACUACUCA-3' (297) and 5'-CCAUCUACUCUGAAGAGAUUGUGGA-3' (383). The sequence of siRNA oligo targeting human p115 is 5'-AAGACCGGCAAUUGUAGUACU-3'. The sequence of siRNA oligo targeting human TGN46 is 5'-CCACCGAAAGCGUCAAGCAAGAAGA-3'

In-solution digestion

Proteins were denatured with 8 M urea in 10 mM Hepes, pH 8.0, and reduced with 10 mM dithiothreitol for 30 min followed by alkylation with 55 mM iodoacetamide for 20 min in the dark. Proteins were digested with 0.5 μg Lys-C (Wako) for 3 h, diluted 1:4 with 10 mM Hepes, pH 8.0, and digested with 0.5 μg trypsin (Promega) for 16 h at room temperature. The digestion was stopped with 0.5% trifluoroacetic acid, 2% acetonitrile. Peptides were desalted on reversed phase C_{18} StageTips (Rappsilber et al., 2003). Directly before analysis, peptides were eluted twice from StageTips using

20 μl of 80% acetonitrile in 0.5% acetic acid. The volume was reduced to 1 μl in a SpeedVac and the peptides were acidified with 5 μl of 2% acetonitrile, 0.1% trifluoroacetic acid in 0.5% acetic acid.

LC MS/MS analysis

A nanoflow HPLC instrument (Easy nLC, Thermo Fisher Scientific) was coupled on-line to an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ion source (Thermo Fisher Scientific). Chromatography columns were packed in-house with ReproSil-Pur C_{18}-AQ 3 μm resin (Dr. Maisch GmbH) in buffer I (0.5% acetic acid). 5 μL of peptides was loaded onto a C_{18}-reversed phase column (15 cm long, 75 μm inner diameter) and separated with a linear gradient of 5–60% buffer II (80% acetonitrile in 0.5% acetic acid) at a flow rate of 250 nL/min over 100 min. MS data were acquired using a data-dependent top10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1650 Th) using CID fragmentation. Survey scans were acquired at a resolution of 60,000 at *m/z* 400. Precursor ion charge state screening was enabled, and all unassigned charge states as well as singly charged species were rejected. Data were acquired using Xcalibur software.

Bioinformatics analysis

Mass spectra were analyzed using MaxQuant software version 1.1.1.22 (Cox and Mann, 2008). The initial maximum allowed mass deviation was set to 6 ppm for monoisotopic precursor ions and 0.5 Da for MS/MS peaks. Enzyme specificity was set to trypsin, defined as C-terminal to arginine and lysine including proline, and a maximum of three missed cleavages was allowed. Carbamidomethylcysteine was set

as a fixed modification, N-terminal acetylation and methionine oxidation as variable modifications. The data were searched against the human and rat International Protein Index protein sequence database (IPI versions 3.68) supplemented with frequently observed contaminants. The required false positive rate was set to 1% at the peptide and 1% at the protein level, and the minimum required peptide length was set to 6 amino acids. Identifications without unique peptides were discarded.

Supplemental figure legends

Supplementary Figure S1

PKD-KD induced tubules contain secretory cargo, but not the late Golgi resident protein sialyltransferase. GST-PKD2-KD and sialyltransferase (ST)-GFP were co-expressed in HeLa cells and visualized by fluorescence microscopy. Arrowheads indicate tubules attached to the TGN. Bar, 10 μm.

Supplementary Figure S2

CARTS are separated from COPI-, COPII-, and clathrin-coated vesicles. PAUF-MycHis was expressed in HeLa cells. The cells were fixed with paraformaldehyde (top and middle rows) or methanol (bottom row). The fixed cells were stained with anti-β-COP, Sec31, and clathrin heavy chain antibodies for COPI, COPII, and clathrin coat protein, respectively. High magnifications of small punctate elements are shown in the inset. Bars, 10 and 5 μm (inset).

Supplementary Figure S3

CARTS contain Rab6, but not ERGIC-53, EEA1, TfR, Lamp-1, M6PR, γ-adaptin, or GGA1. PAUF-MycHis were expressed in HeLa cells. The cells were fixed with paraformaldehyde, incubated with specific antibodies, and visualized by fluorescence microscopy. High magnifications of small punctate elements are shown in the inset. Bars, 10 and 5 μm (inset).

Supplementary Figure S4

Ultrastructure of CARTS. HeLa cells were transfected with a plasmid for PAUF-MycHis and on the following day they were incubated at 20°C for 5 h in the presence of cycloheximide. The cells were shifted to 37°C to release PAUF-MycHis from the Golgi membranes and fixed after 60 min. Bar, 200 nm.

Supplementary Figure S5

Disruption of myosin II function does not affect the biogenesis of CARTS. (A-D) HeLa cells were transfected with control (Cont) siRNA or siRNA oligos specific for myosin IIa. Forty-eight hours later, the cells were transfected with a plasmid for PAUF-MycHis. Seventy-two hours after siRNA transfection, the cells were lysed or fixed for fluorescence microscopy. (A) The cell lysates were western blotted with anti-myosin II antibody and anti-calnexin antibody as a control. (B) Quantification of the expression levels of myosin II upon RNAi. The average values of four independent experiments are shown. (C) The fixed cells were stained with anti-Myc antibody. Bar, 10 μm. (D) Quantification of CARTS formation. The number of CARTS in control cells (n=5) and myosin II knockdown cells (n=5: siRNA (297), n=3: siRNA (383)) was counted. The average number of CARTS per cell is shown. (E, F) The starting materials, permeabilized HeLa cells and rat liver cytosol were

separately preincubated with DMSO (control) and 100 μM blebbistatin (Bleb) for 10 min and then mixed in the presence of an ATP regenerating system for 45 min. The high speed pellet containing CARTS was western blotted with anti-TGN46 antibody. (F) Quantification of CARTS formation. The average values of two independent experiments are shown.

Supplementary Figure S6

p115 is not required for PAUF secretion. (A-D) HeLa cells were transfected with control (Cont) siRNA or siRNA specific for p115. Two days later, the cells were transfected with a plasmid for PAUF-MycHis and the next day cells were incubated at 20°C for 2 h. After shifting to 32°C for 1 h the medium was collected to monitor PAUF-MycHis secretion, and the cells were lysed to test the efficiency of p115 knockdown. (A) Knockdown efficiency of p115 was monitored by western blotting the cell lysate using anti-p115 and anti-β-actin antibodies. (B) Quantification of the expression levels of p115 upon RNAi. The average values of three independent experiments are shown. (C) The secretion of PAUF-MycHis was monitored by western blotting the cell lysate and the medium using anti-His antibody. (D) Quantification of PAUF secretion. The amount of secreted PAUF was normalized with the expression level. The average values of four independent experiments are shown.

Supplementary Figure S7

CARTS are distinct from carriers that transport collagen I or VSV-G. (A) PAUF-MycHis and GFP-collagen I expressing HeLa cells were incubated at 20°C for 2 h in the presence of 20 μg/ml cycloheximide and then shifted to 32°C for 15 min. The

cells were stained with anti-Myc and Sec31 antibodies for PAUF-MycHis and COPII coat protein, respectively. (B) PAUF-MycHis and VSV-G-GFP expressing HeLa cells were incubated at 40°C overnight, after which they were incubated at 20°C for 2 h in the presence of 20 μg/ml cycloheximide and then shifted to 32°C for 0, 5, 15, or 60 min. The cells were visualized with fluorescence microscopy. High magnifications of small punctate elements are shown in the inset. Bars, 10 and 5 μm (inset).

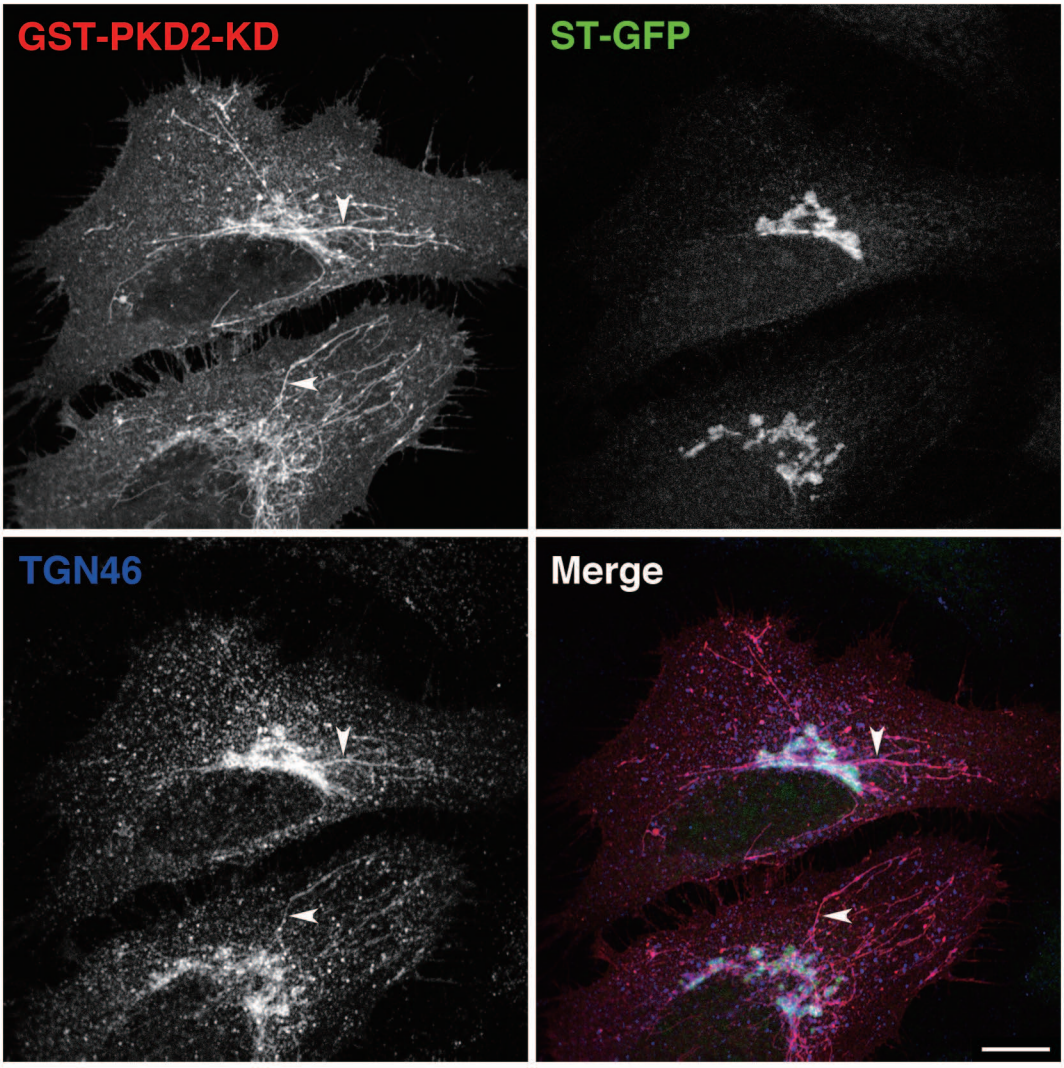
Supplementary Movie S1

CARTS trafficking. HeLa cells expressing PAUF-mRFP were imaged at 440 ms intervals for approximately 3 min. Arrows indicate PAUF containing membranes, which disappear presumably by fusion with the cell surface.

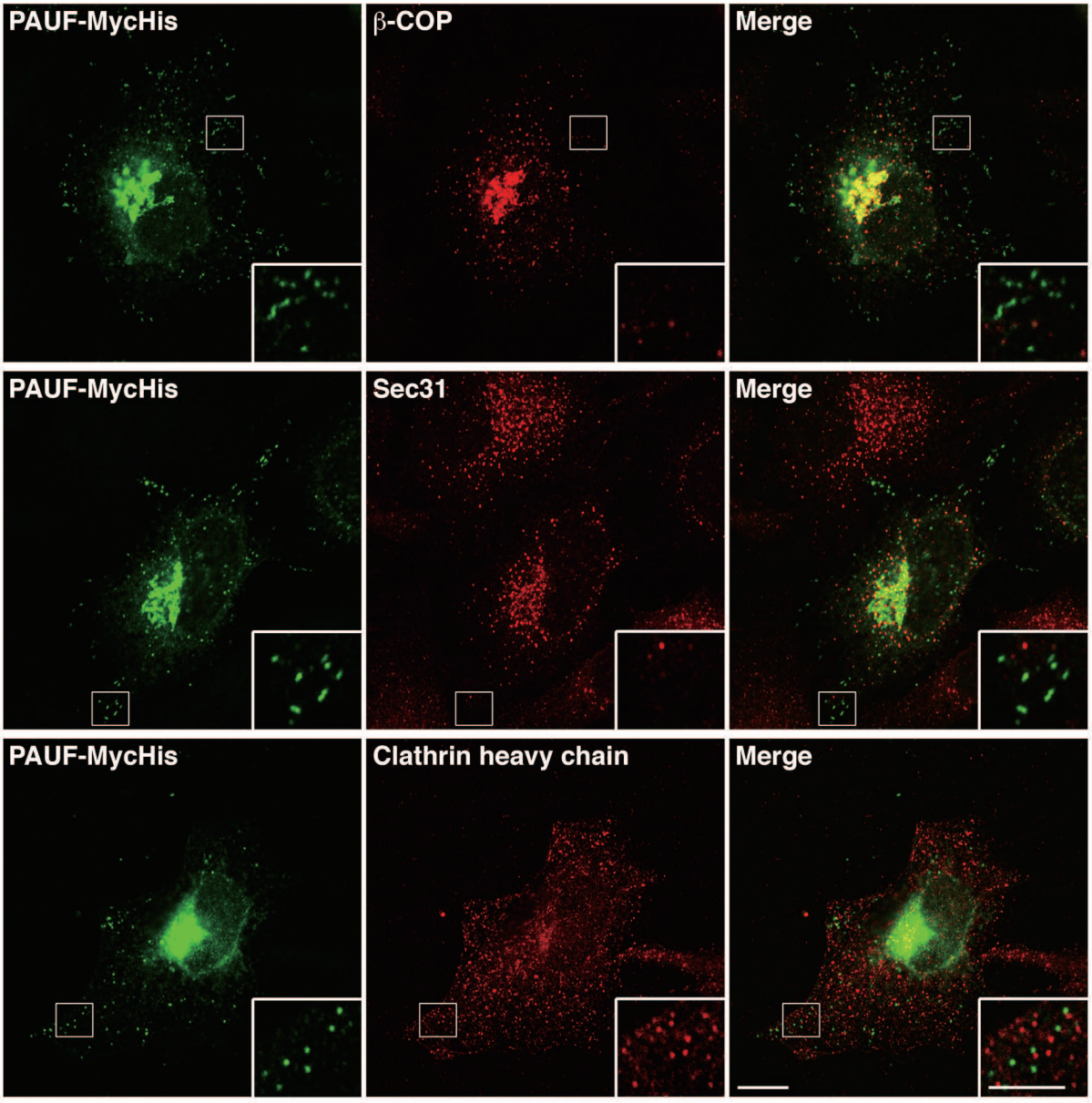
Supplementary Table SI

Complete polypeptide composition of the TGN46 containing transport carriers. Since HeLa cells and rat liver cytosol were used for the transport carrier formation, the data of the mass spectrometric analysis were searched against human and rat protein sequence databases. The number of unique peptides is indicated between brackets. Common sequences of human and rat origin can be found in both databases. The proteins highlighted in blue are shown in Table I as the most likely components of CARTS.

Supplementary figure 1.



Supplementary figure 2.



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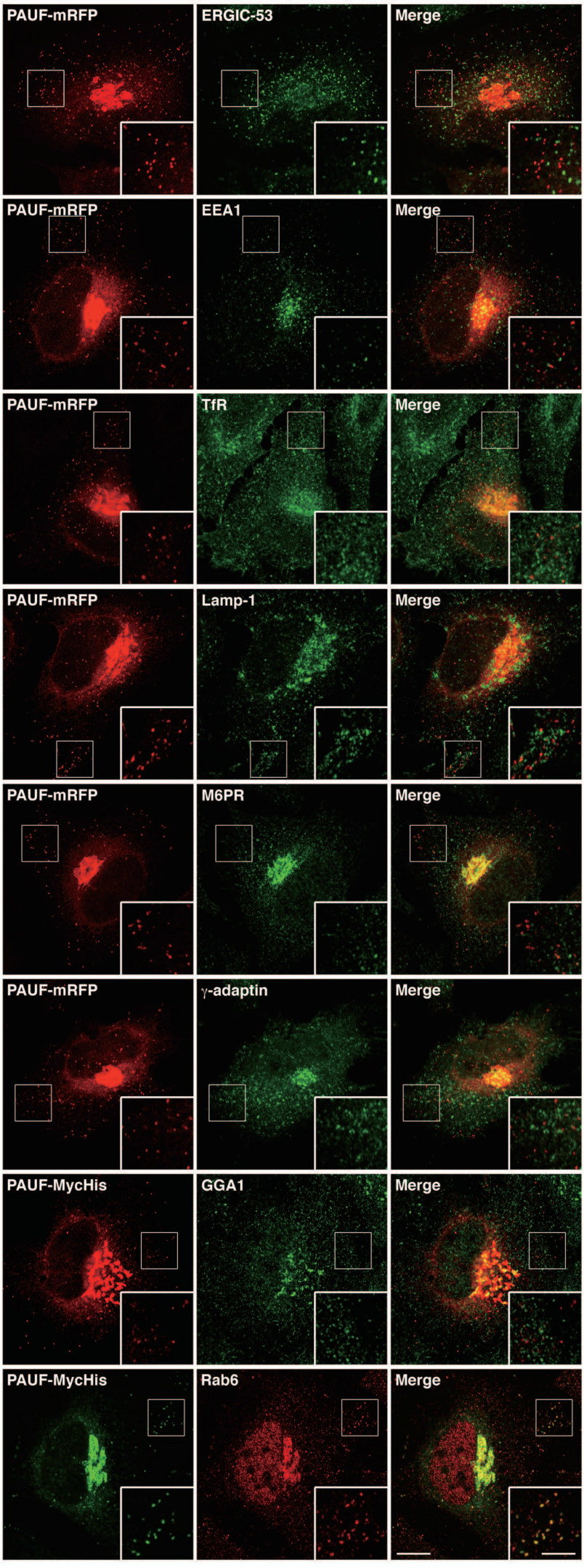

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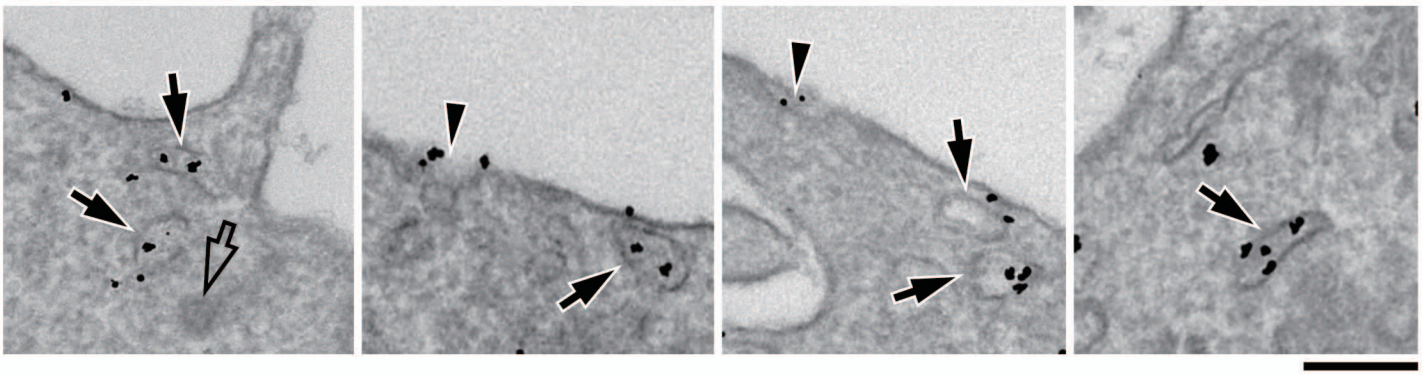

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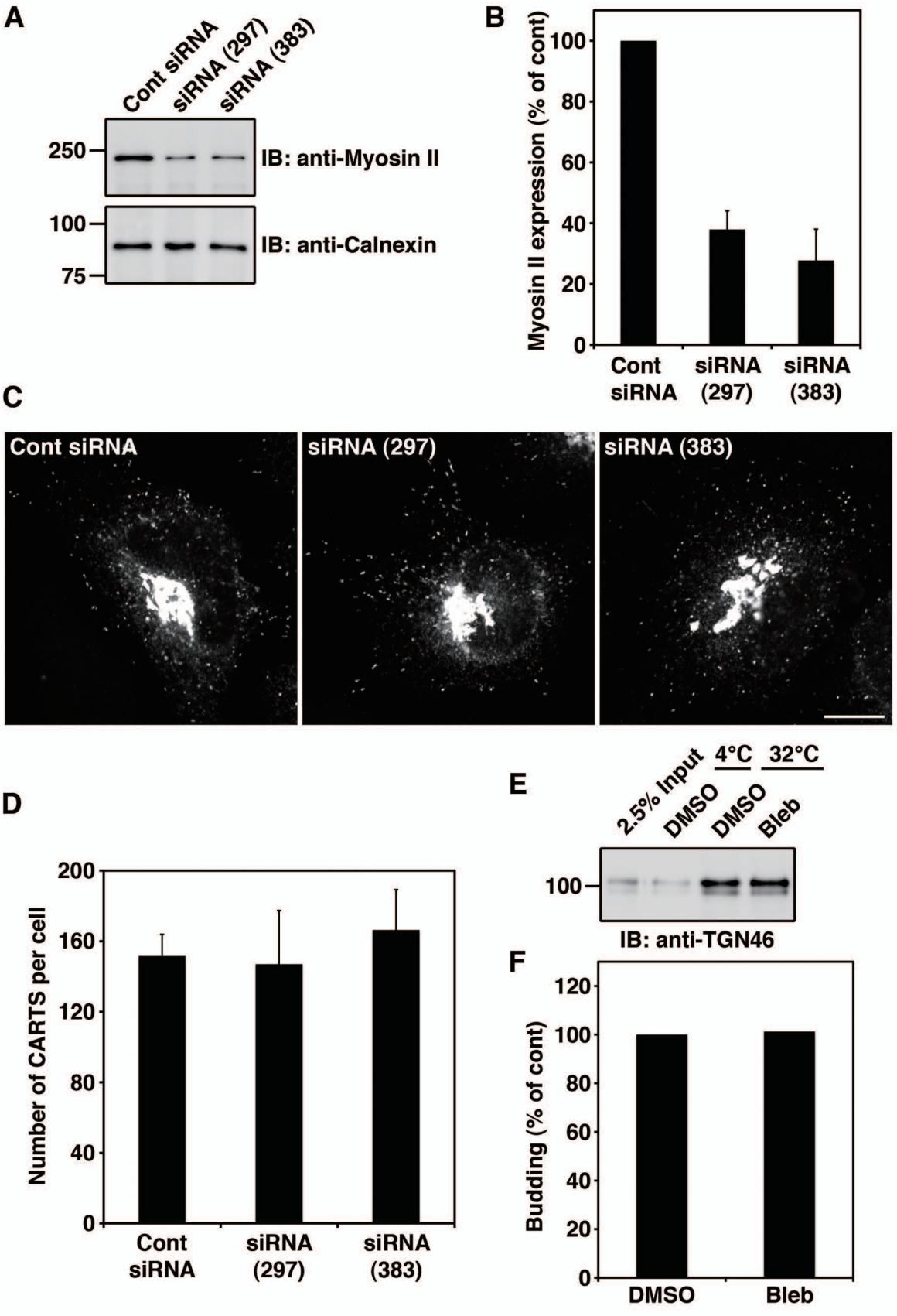
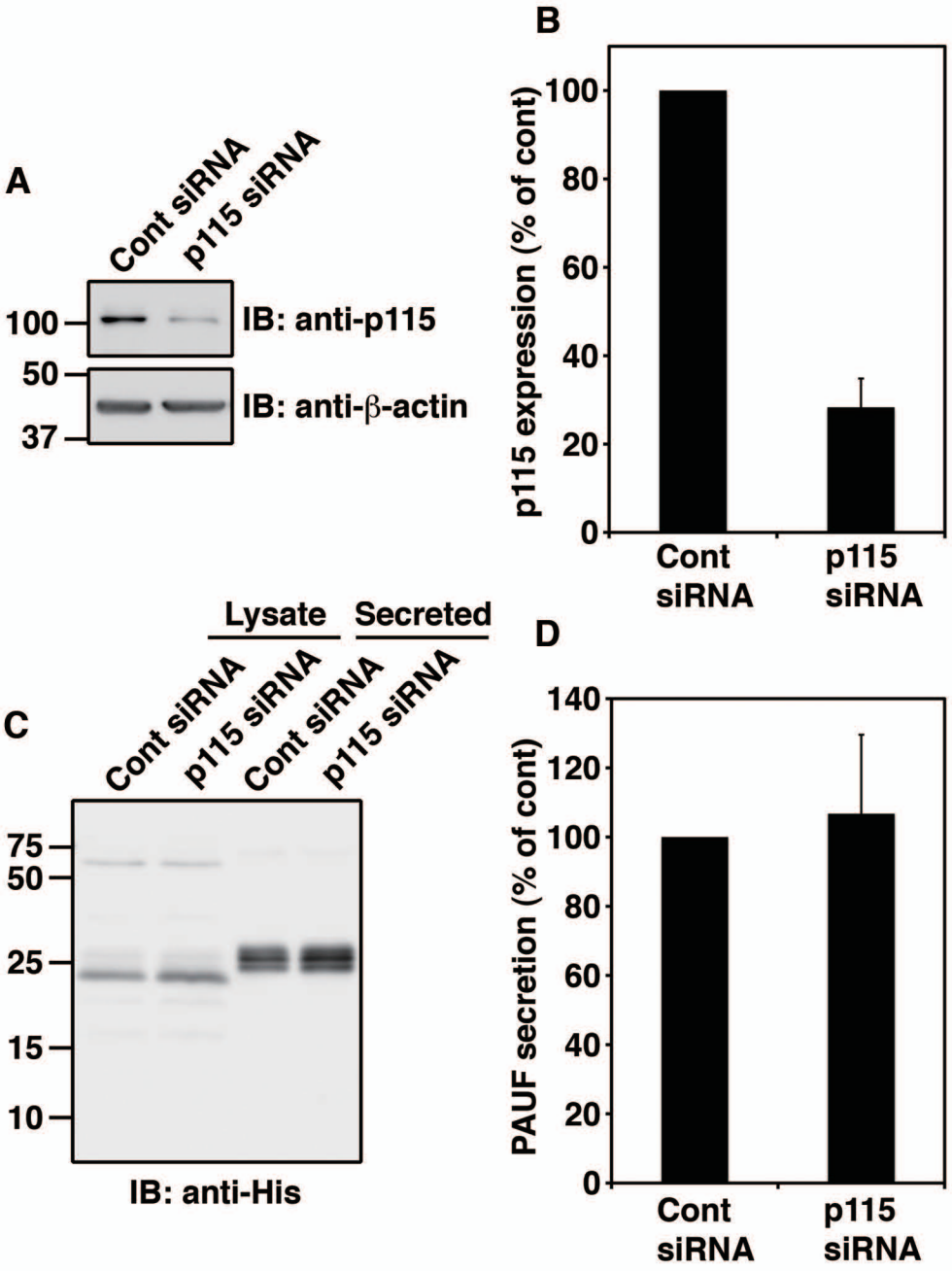


Supplementary figure 6.



Supplementary figure 7.