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RNA interference-mediated silencing of the syntaxin 5 gene induces Golgi fragmentation but capable of transporting vesicles

Kei Suga*[,1], Hiroshi Hattori[1,2], Ayako Saito, Kimio Akagawa

Department of Cell Physiology, Kyorin University School of Medicine, Mitaka, Tokyo 181-8611, Japan

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Abstract It has been suggested that syntaxin 5 (Syx5) participates in vesicular transport. We examined the effects of Syx5 down-regulation on the morphology of the Golgi apparatus and the transport of vesicles in mammalian cells. Knockdown of the Syx5 gene resulted in Golgi fragmentation without changing the level of endoplasmic reticulum (ER)-resident proteins, other Golgi-SNAREs (soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptors), and coatmer proteins. Strikingly, a major decrease in Syx5 expression barely affected the anterograde transport of vesicular stomatitis virus G (VSVG) protein to the plasma membrane. These results suggest that Syx5 is required for the maintenance of the Golgi structures but may not play a major role in the transport of vesicles carrying VSVG between the ER and the Golgi compartment.
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1. Introduction

Many members of the mammalian syntaxin (Syx) family have been identified; most isoforms localize to specific membrane compartments along the secretory and endocytotic pathways and are thought to be involved in the intracellular trafficking of vesicles (reviewed by [1]). The first group of Syxs identified [Syx1A (HPC-1), 2, 3, and 4] predominantly localize to the plasma membrane; these Syxs may mediate constitutive and regulated exocytotic events at the cell surface [2–6]. Syx5 is resident in the endoplasmic reticulum (ER)-to-Golgi compartment of the early secretory pathways [7–9] and has two isoforms (42 and 35 kDa) that are generated from a single messenger RNA through alternative translation in mammalian cells [10]. The 42-kDa form (designated Syx5L) has an amino-terminal extension containing a type-II ER retrieval signal and preferentially localizes to the ER, whereas the 35-kDa form (Syx5) localizes to the *cis*-Golgi network. Syx5 is thought to regulate the potential targeting and/or fusion of carrier vesicles at three different membrane fusion interfaces: the fusion of ER-derived vesicles with transitional ER–Golgi intermediate compartment/vesicular tubular carriers (ERGIC/VTC), the assembly of ERGIC/VTCs to form *cis*-Golgi elements, and the fusion in the intra-Golgi compartment by the selective combination of a soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) complex with other SNARE related proteins [7,11–21]. In addition, in vitro reconstitution experiments show that Syx5 may also participate in transport from the early/recycling endosome to the *trans*-Golgi network (TGN) [22]. These results indicate that Syx5 may serve as a target-SNARE (t-SNARE) and play a central role in the transport of membrane vesicles along the secretory and postTGN/endocytotic pathways. Although there have been many reports implying important roles for Syx5 in the transport of vesicles in cell-free systems and in yeast cells, it has not been examined whether Syx5 is indispensable for these functions in mammalian cells. As it seems likely that the organization of the early secretory compartments differs across species [23], the role of Syx5 in mammals has to be explored. In addition, although the precise function is unclear, others and we have recently shown that Syx5 may have roles other than vesicle transport in the cell [24–26].

In this study, in order to investigate the physiological significance of Syx5, we have used the RNA interference (RNAi) method (for review, see [27]) to knockdown the expression of the Syx5 gene in mammalian cells. We focused on the effect of Syx5 knockdown on the structure of the Golgi apparatus and the transport of the vesicular stomatitis virus ts045 G protein. For the first time, using the RNAi method, we demonstrated that Syx5 is required for the maintenance of the structures of the Golgi apparatus but may not play a central role in the anterograde transport of membrane proteins along the secretory pathway.

2. Materials and methods

2.1. Materials and antibodies

Polyethylene glycol and rat monoclonal anti-hemagglutinin (HA) antibody (3F10) were purchased from Roche Diagnostics (Indianapolis, IN, USA). TiterMax Gold was from CytRx Corporation (Norcross, GA, USA). Murine monoclonal antibodies against BiP/GRP78, GM130, and γ-Adaptin were from BD Transduction Laboratories (San Diego, CA, USA). Mouse monoclonal anti-α-tubulin antibody

*Corresponding author. Fax: +81 0422 47 4801.
E-mail address: ksuga@kyorin-u.ac.jp (K. Suga).

[1] These authors contributed equally to this work.
[2] Present address: Toyama Chemical Co., Ltd., 2-4-1 Shimo-okui, Toyama 930-8508, Japan.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; ERGIC/VTC, ER–Golgi-intermediate compartment/vesicular tubular carriers; GFP, green fluorescent protein; GST, glutathione-*S*-transferase; HA, hemagglutinin; mAb, monoclonal antibody; RNAi, RNA interference; siRNA, small interfering RNA; SNARE, soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor; Syx, syntaxin; VSVG, vesicular stomatitis virus ts045 G

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was from Sigma (St. Louis, MO, USA). Murine monoclonal anti-GS28 and anti-Membrin antibodies were from Stressgen (Victoria, BC, Canada). Rabbit polyclonal anti-βCOP, anti-EEA1, and anti-LAMP1 antibodies and murine monoclonal anti-SERCA2 antibody were from Affinity Bioreagents (Golden, CO, USA). Alexa Fluor 488 anti-mouse, anti-rat, and anti-rabbit IgGs were purchased from Molecular Probes (Eugene, OR, USA). The Cy3-labelled anti-rat and anti-mouse IgGs were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Hoechst 33342 and Brefeldin A (BFA) were purchased from Sigma. BFA was stored as a stock solution at 5 mg/ml methanol. The protease inhibitor cocktail was purchased from Wako Chemicals (Osaka, Japan). All other reagents were of the highest grade available, unless otherwise noted.

2.2. Plasmids and duplex siRNA

Expression plasmids for the cDNAs encoding the HA-tagged full-length human syntaxins 1A, 2, 3, 4, 5, 6, 7, and 8 (h1, h2, h3, h4, h5, h6, h7, and h8) and chimeric mutants of Syx5 and Syx1A were described previously [25,26]. The cloning of the cDNAs encoding the long-splicing isoform of Syx5 (designated as Syx5L) was performed by generating oligonucleotide primers based on the sequence of cDNA clone DKFZp313I1240 (EMBL Accession No. BX537426). The digested *Bam*HI–*Xho*I fragments that contained full-length Syx5L were inserted into the *Bam*HI–*Xho*I site of the pcDNA3-HAN vector in frame to produce h5L-pCDNA3-HAN. The digested *Bam*HI–*Not*I fragment that contained a cDNA fragment for aa 1–333 of Syx5L was inserted into the *Bam*HI–*Not*I site of pGEX5X-3 vector in frame to generate h5LΔTM-pGEX5X-3. A vesicular stomatitis virus ts045 G (VSVG)-green fluorescent protein (GFP)-expression plasmid was kindly provided by Dr. Jennifer Lippincott-Schwartz (Cell Biology and Metabolism Branch, NICHHD, NICHD, NIH.). A 19-base pair (bp) sequence for Syx5 (nucleotides 270–288) was selected from the human Syx5 sequence. The small interfering RNAs (siRNAs) corresponding to the Syx5 sequence and its scrambled sequence with an additional two-base 3′ overhang (TT) on each strand were obtained from Ambion (Austin, TX, USA). The sequence of Syx5 siRNA was: 5′-UAGCCUCAACAAACAAAUUTT-3′ for the sense and 5′-AAUUUGUUUGUUGAGGCUATT-3′ for the antisense strands. The sequence of the scrambled siRNA was: 5′-UGUACAAAUCA-CACCAUAATT-3′ for the sense and 5′-UUAUGGUGUGAUUU-GUACATT-3′ for the antisense strands. The annealing of duplex siRNAs was performed according to the manufacturer's protocol. An expression vector for Syx5 siRNA was constructed using a Silencer™Express kit (Ambion). Briefly, oligonucleotides containing sense and antisense strands of the Syx5 sequence (nucleotides 270–288), loop region, RNA polymerase III promoter and terminator sequences, and *Bam*HI and *Hin*dIII restriction enzyme sites were chemically synthesized, annealed, and cloned into the *Bam*HI and *Hin*dIII sites of p*Silencer* 3.1-H1 neo vector. The p*Silencer* 3.1-H1 neo vector was used as a negative control. The plasmids for the deletion mutants of Syx5 fused to glutathione-*S*-transferase (GST) [h5ΔTM (1–279), h5ΔH3-pGEX5X-3 (1–204), h5Habc-pGEX5X-3 (1–158), h5Δhab3-pGEX5X-3 (113–204), h5Hab-pGEX5X-3 (1–112), h5ΔHabc-pGEX5X-3 (159–279), and h5H3-pGEX5X-3 (204–279)] were described previously [26]. Additional plasmids, h5ΔHa3-pGEX5X-3 (66–204), h5Hbc-pGEX5X-3 (66–158), h5Hc-pGEX5X-3 (113–158), h5Hinge-pGEX5X-3 (159–204), h5Ha-pGEX5X-3 (66–279), and h5ΔHab-pGEX5X-3 (113–279), were constructed in a similar manner. The identities of the inserted sequences were verified for the constructs, both by direct sequencing using the ABI373A sequencer with the Big-Dye filter (Applied Biosystems) and by digestion with the appropriate restriction enzymes.

2.3. Cell culture and transfection

PC12, HeLa, COS-7, and NG108-15 cells were cultured as described previously [25,26,28]. The cells were inoculated into 60-mm dishes for Western blotting or 35-mm dishes with glass bottoms for the immunocytochemical analyses. The cells were transfected with the duplex siRNA or the siRNA expression plasmid using Lipofectamine 2000 transfection reagent (Invitrogen, Groningen, The Netherlands) or FuGene6 transfection reagent (Roche Diagnostics), respectively. Two days after transfection, the cells were subjected to Western blotting or immunocytochemical analysis.

2.4. Expression and purification of GST-fusion proteins

To express the recombinant GST-fusion proteins, plasmids encoding GST-fused deletion mutants of Syx5 were transformed into *Escherichia coli* strain BL21 (Stratagene). The purification of GST-fusion proteins from bacterial lysates was performed as described previously [26,28].

2.5. Production of monoclonal antibodies against Syx5

The purified GST-h5ΔTM was used for immunization. Six-to-eight-week-old BALB/c mice (Clea Japan, Tokyo, Japan) were immunized with purified GST-h5ΔTM (20 μg protein/mouse) in buffer containing 1% SDS emulsified with an equal volume of TiterMax Gold by i.p. injection three times at 2-week intervals. Spleen cells were fused with PAI mouse myeloma cells using the polyethylene glycol method as described previously [28]. The hybridomas were selected in medium containing 2.5 mM hypoxanthine, 10 μM aminopterin, 0.4 mM thymidine, and were cultured in RPMI-1640 medium containing 20% fetal calf serum (FCS). Culture supernatants from the hybridoma cultures were screened by enzyme-linked immunosorbent assay (ELISA), Western blotting, and an indirect immunofluorescent method. Purified GST-fusion proteins of Syx5 (Fig. 1A) and lysates from HeLa cells were used as antigens for the ELISA and Western blotting. Positive hybridoma cells were cloned from a single clone that was derived by the limiting dilution method. Antibody classes were identified with the Mouse Monoclonal Antibody Isotyping kit RPN29 (Amersham Biosciences). Ascites fluid was produced from BALB/c mice that had been implanted intraperitoneally with the hybridoma. The IgG fraction was obtained by affinity chromatography on protein G–Sepharose 4FF (Amersham Biosciences) as described previously [28].

2.6. Extract preparation, SDS–PAGE, and Western blotting

Cells transfected with duplex siRNA were harvested with a cell scraper, recovered by centrifugation, lysed in extraction buffer (50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, and 1% Triton X-100) containing a protease inhibitor cocktail, and then sonicated for 30 s. The insoluble material was removed by centrifugation at $15{,}000 \times g$ for 10 min, and the supernatant was used as the extract sample. The samples were subjected to SDS–PAGE followed by Western blotting as described previously [25,28].

2.7. Immunocytochemical analysis

Immunocytochemical analyses were carried out essentially as described previously [25,26].

2.8. VSVG transport assay

Cells were plated on a 35-mm dish with a glass bottom and transfected with 30 nM of duplex siRNA. Twenty-four hours after transfection, the cells were transfected with the VSVG-GFP-expression plasmid and incubated for 14–16 h at 40 °C. The cells were then incubated for the indicated times at 32 °C and subjected to immunocytochemical analyses. In the case of Syx5 overexpression, the cells were co-transfected with h5-pcDNA3-HAN and the VSVG-GFP-expression plasmid and incubated for 14–16 h at 40 °C prior to the temperature shift to 32 °C. The inhibition by BFA of VSVG-GFP transport from ER to the Golgi compartment was confirmed by a time-lapse imaging experiment. In brief, cells grown in a 35-mm dish with a glass bottom were supplemented with Hank's Balanced Salt Solution containing 20 mM HEPES, pH 7.4, and 0.05% BSA (HHB buffer), and the temperature was maintained at 32 °C. The cells were visualized under an inverted microscope (IX70, Olympus) with a 60× PlanApo lens (numerical aperture, 1.4 oil) using a standard filter set. Sequential images were acquired with a cooled CCD camera (CoolSNAP, Roper Scientific, Trenton, NJ, USA) under the control of Metamorph software (Universal Imaging, Downingtown, PA, USA). BFA treatment was performed by applying HHB buffer containing BFA to give a final concentration of 10 μg of BFA per ml in a culture dish placed on the stage.

3. Results

3.1. Generation and epitope mapping of a mAb specific for Syx5

In order to examine the physiological role of Syx5, we first produced the specific monoclonal antibody 1C5 to Syx5. Syx5 is

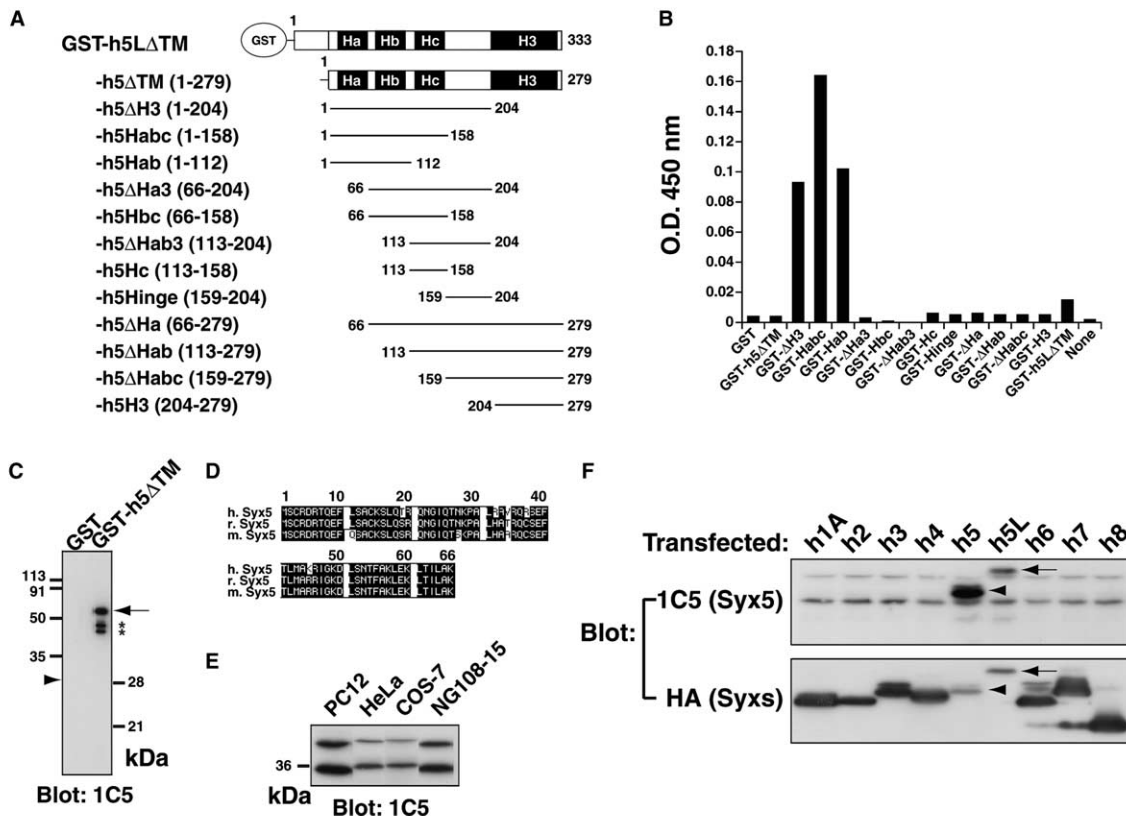

Fig. 1. Characterization of a monoclonal antibody specific for Syx5. (A) Schematic of the GST-fusion proteins of the Syx5L and Syx5 transmembrane deletion mutants. The putative α-helices (Ha, Hb, Hc, and H3 domains) of Syx5 are indicated in black. The putative transmembrane region of Syx5 (aa 280–301) is omitted. Recombinant GST-h5ΔTM protein was expressed in *E. coli*, purified, and used for immunization and screening of hybridomas by ELISA under non-denaturing conditions and by Western blot analysis under SDS-denaturing conditions. The numbers are those of amino acid residues. (B) Epitope mapping of mAb 1C5 by ELISA under non-denaturing conditions using the various truncation mutants of Syx5 shown in (A). The culture supernatant of hybridoma producing mAb 1C5 was used, and the absorbance at 450 nm is presented. Note that polypeptides containing amino acid residues 1–66 reacted with 1C5. (C) Purified GST and GST-h5ΔTM protein were subjected to SDS–PAGE followed by immunoblotting. Note that, under SDS-denaturing conditions, the mAb 1C5 reacted with GST-h5ΔTM but not with GST. (D) An alignment of amino acid sequences containing the 1C5 epitope in human (h), rat (r), and mouse (m) Syx5. NCBI accession numbers are as follows: Q13190(hSyx5), F48213 (rSyx5), and Q8K1E0 (mSyx5). (E) Western blot analyses were performed with endogenous Syx5 expressed in various cell lines. Total cellular protein (40 μg) extracted from a given cell line (as indicated) was subjected to immunoblotting. Note that the mAb 1C5 recognizes rat, human, monkey, and mouse Syx5 isoforms. (F) Specificity of mAb 1C5 for Syx5. HeLa cells were transfected with various human Syxs and subjected to immunoblotting. The gels are representative of those obtained from at least three independent experiments. Arrows and arrowheads indicate expressed HA-tagged Syx5L and Syx5, respectively. Endogenous Syx5 and Syx5L were labelled in all lanes (h1A-h8) by mAb 1C5. The mAb 1C5 specifically recognized Syx5 and Syx5L but does not react with the other Syxs.

classified as a tail-anchored protein with four α-helices (the Ha, Hb, Hc, and H3 domains) and a putative C-terminal membrane anchor region (Fig. 1A). For the epitope mapping of 1C5, various truncation mutants of GST-fused Syx5 were generated (Fig. 1A). ELISA analysis using purified GST-fusion proteins of Syx5 revealed that the epitope of 1C5 maps to aa 1–66 from the N-terminus (Fig. 1B). Although 1C5 did not react with GST-h5ΔTM in the non-denatured condition (ELISA analysis), Western blotting analyses under denaturing conditions revealed that 1C5 does react with GST-h5ΔTM (Fig. 1C). The reactivity of 1C5 to the N-terminal region (aa 1–66) of GST-fused Syx5 was confirmed by Western blot analyses (data not shown). In addition, 1C5 reacted with Syx5L under non-denaturing (ELISA analysis in Fig. 1B) and denaturing conditions (Western blot analysis, data not shown). As Syx5 is highly conserved among mammals (Fig. 1D), we examined the cross-reactivity of 1C5 to Syx5 of other species. As shown in Fig. 1E, the results from Western blotting showed that 1C5 recognizes Syx5 of humans, rat, mouse, and monkey. To further establish the specificity of monoclonal antibody (mAb) 1C5, various human Syxs were expressed, and the reactivity of 1C5 to other Syxs was analyzed. As shown in Fig. 1F, 1C5 recognized Syx5 and Syx5L but not the other Syxs studied so far. These results demonstrate the specificity of mAb 1C5 for the N-terminal region containing the Ha domain of Syx5 of various species.

3.2. Knockdown of syntaxin 5 by RNAi method

To reduce the amount of endogenous Syx5 in cells, an RNAi technique was used. Two different target sequences were synthesized and tested for the efficiency of Syx5 knockdown by immunoblotting and immunocytochemical analyses. As shown in Fig. 2A, one of the siRNAs efficiently decreased the amount of endogenous Syx5s in HeLa cells. The dose-dependent inhibition of the expression of Syx5s was clearly observed by immunoblotting. Densitometric analysis showed that, when 30 nM of duplex siRNA was used, the amount of Syx5s was decreased to less than 15% of that in control cells. It is noteworthy that both short and long isoforms of Syx5

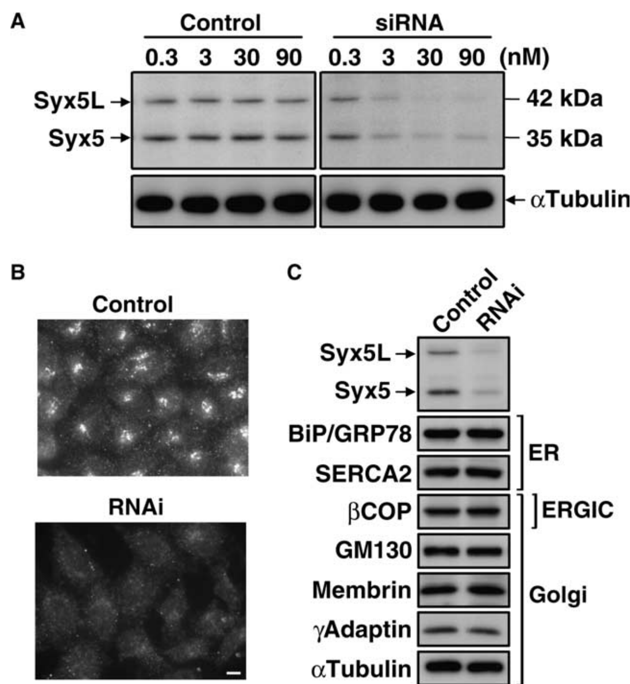

Fig. 2. Knockdown of Syx5 by RNAi. (A) HeLa cells were transfected with the indicated doses of duplex siRNA for Syx5 or the scrambled duplex. Two days after transfection, the cells were extracted and subjected to Western blotting with a mAb to Syx5 (1C5). Knockdown by siRNA dose-dependently decreased the amount of Syx5 protein. (B) Knockdown of Syx5 was assessed by an indirect immunofluorescence method using mAb 1C5 antibody. Immunoreactivity of Syx5 is absent in knockdown cells. (C) Specific knockdown of Syx5. Syx5 knockdown cells (transfected with 30 nM siRNA) were subjected to Western blotting with the indicated marker proteins for the ER, ERGIC/VTC, and Golgi apparatus. BiP/GRP78 and SERCA2 were used as markers for the ER; βCOP was used for the ERGIC and Golgi apparatus; GM130 was used for the *cis*-Golgi network; Membrin was used for the Golgi apparatus; and γ-Adaptin was used for the *trans*-Golgi network. α-Tubulin was used as a loading control. The gels and images are representative of those obtained from at least three independent experiments. Syx5 RNAi did not affect the expressed amounts of representative marker proteins of the ER and Golgi compartments. Bar, 10 μm.

(Syx5 and Syx5L, respectively) were equally down-regulated (Fig. 2A, right panel). The amount of remaining Syx5 proteins in cells transfected with siRNA expression-plasmid was decreased to approximately 35% of that in control vector transfected cells. The immunocytochemical analysis shown in Fig. 2B demonstrated that Syx5s resident in the vesicles and the Golgi apparatus (as can be seen in control cells) were diminished in the RNAi-induced cells.

Selective knockdown of Syx5 with specific siRNA was confirmed by immunoblotting. We examined the amounts of proteins located in the ER, the ER–Golgi-intermediate-compartment (ERGIC/VTC), and the Golgi apparatus in Syx5 knockdown cells (Fig. 2C). As assessed by the marker for the ER (BiP/GRP78, SERCA2), the marker for the ERGIC/VTC and Golgi apparatus (βCOP), the marker for the *cis*-Golgi network (GM130), and the marker for the *trans*-Golgi network (γ-Adaptin) [29,30], the expression of Syx5 siRNA did not change the cellular levels of representative proteins from the ER through the Golgi apparatus. It is important to note that the expression level of Membrin (a binding partner of Syx5) was not changed by the down-regulation of Syx5s. Similar results were also found in cells transfected with the siRNA-expression plasmid (data not shown).

Next, we examined the effect of Syx5s overexpression on the amounts of marker proteins in the ER through the Golgi apparatus. HeLa cells were transfected with either HA-tagged Syx5 or Syx5L and analyzed by immunoblotting. Similar to the levels in knockdown cells, the cellular levels of the representative proteins from the ER through the Golgi apparatus were not affected (data not shown). The change in the amount of Syx5 was protein-specific, as cellular levels of the tested marker proteins for the ER, ERGIC/VTC, and Golgi apparatus were not altered in HeLa cells.

3.3. *Effect of syntaxin 5 knockdown and overexpression on structures of the Golgi apparatus*

Although the alteration of the amount of Syx5 had no effect on the cellular expression of the marker proteins for the ER, ERGIC, and Golgi apparatus, it might affect their specific intracellular localization. Therefore, we investigated immunocytochemically the intracellular localization of the proteins (used in Fig. 2) in cells in which Syx5 was knocked down or overexpressed. As shown in Fig. 3A, the intracellular localization of SERCA2 (a marker for the ER) did not differ in cells treated with the siRNA expression plasmid. However, the immunoreactivity of βCOP, GM130, Membrin, GS28, and γ-Adaptin, all of which reside in the Golgi apparatus, were scattered. It is noteworthy that the localization of the Golgi SNAREs Membrin and GS28 were altered by Syx5 knockdown. To confirm that these changes in localization were attributable to Syx5 down-regulation, cells were doubly stained with 1C5 and anti-βCOP antibody. We found that βCOP was dispersed as a vesicular structure in more than 95% of the cells in which Syx5 was down-regulated (Fig. 3B, upper panels). So, it was obvious that all most all the Syx5 knockdown cells revealed dispersed βCOP immunoreactivity. Given that βCOP reflects the localization of the COPI vesicle, we investigated the correlation between βCOP (and Syx5) localization and the structure of the Golgi apparatus by using the Golgi matrix protein GM130, which functions as a tethering factor at the face of the *cis*-Golgi [30]. As shown in the bottom panels in Fig. 3B, over 95% of the cells that showed dispersed βCOP were associated with the dispersion of GM130 immunoreactivity. Similarly, other proteins that reside in the Golgi apparatus (Membrin, GS28, and γ-Adaptin) were also scattered when colocalization were examined with βCOP (data not shown). In agreement with the results obtained in HeLa cells, similar dispersion of βCOP and GM130 induced by the down-regulation of Syx5 was observed in NG108-15 cells and in cultured hippocampal neurons (data not shown). However, Syx5 knockdown did not alter the intracellular localization of early-endosomal antigen 1 (EEA1) and the lysosome associated membrane protein 1 (LAMP1) indicating that Syx5 depletion may mot affect post-TGN, endosomal, and lysosomal systems (data not shown). These results suggest that Syx5 depletion causes the fragmentation of the Golgi apparatus but the tethering of COPI vesicles is normal, at least in the *cis*-Golgi network.

We also investigated the effects of Syx5 overexpression on the intracellular localization of βCOP and GM130. In cells in which Syx5 was overexpressed (Fig. 3C), βCOP was dispersed as vesicular structures and was associated with the fragmentation of the Golgi apparatus as assessed by GM130. These results suggest that the amount of Syx5 is critical for the maintenance and/or organization of the structure of the Golgi apparatus.

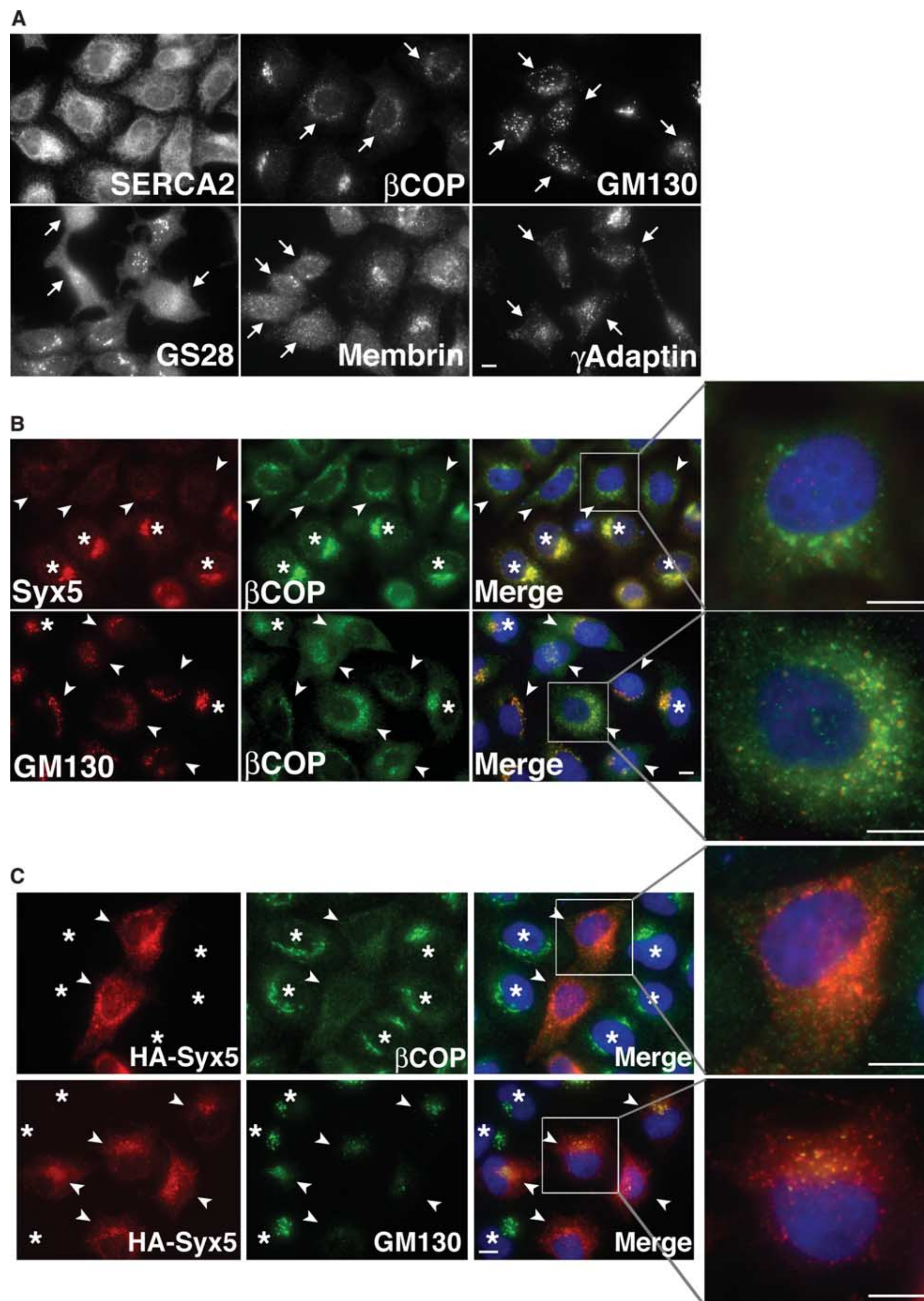

Fig. 3. Effect of Syx5 knockdown and overexpression on the structure of the ER and Golgi apparatus. (A) HeLa cells were transfected with an siRNA-expression plasmid and subjected to immunocytochemistry with the indicated antibodies. While the localization of the ER marker (SERCA2) was normal, the markers representing the Golgi apparatus (βCOP, GM130, GS28, Membrin, and γ-Adaptin) showed dispersed morphology of Golgi in the cells indicated by arrows. (B) Cells transfected with duplex siRNA were subjected to double-immunofluorescence immunocytochemistry with anti-βCOP antibody and 1C5 or anti-GM130 antibody. Hoechst33342 staining is shown in blue. Representative images are shown. The merged images demonstrate the co-localization of βCOP with Syx5 and GM130, and the fragmentation of the Golgi apparatus in Syx5 knockdown cells (arrowheads). (C) Cells transfected with HA-tagged Syx5-expressing plasmid were subjected to double-immunofluorescence immunocytochemistry. Overexpressed Syx5 was detected with anti HA antibody (3F10) and was stained with antibody for βCOP or GM130. Representative images are shown. Arrowheads indicate the Syx5-overexpressing cells showing the dispersion of βCOP-positive vesicles (upper panels) and the fragmentation of the Golgi apparatus (bottom panels). The cells with asterisks indicate cells which have morphologically normal βCOP localization and Syx5 expression appeared to be unchanged in these cells. Images in the right columns show magnified views of boxed areas. Bars, 10 μm.

3.4. *Effects of syntaxin 5 knockdown on vesicular transport*

Syx5 has been suggested to act as a t-SNARE involved in vesicular transport between the ER and Golgi. Therefore, we examined the effects of Syx5 on the transport of a temperature-sensitive mutant of vesicular stomatitis virus ts045-G protein (VSVGts045) as a model transmembrane glycoprotein that is normally trafficked along the secretory pathways [31,32]. As has been reported [33,34], the treatment of cells with a fungal toxin, BFA, that specifically inhibits the transport of vesicles from the ER to the Golgi apparatus led to

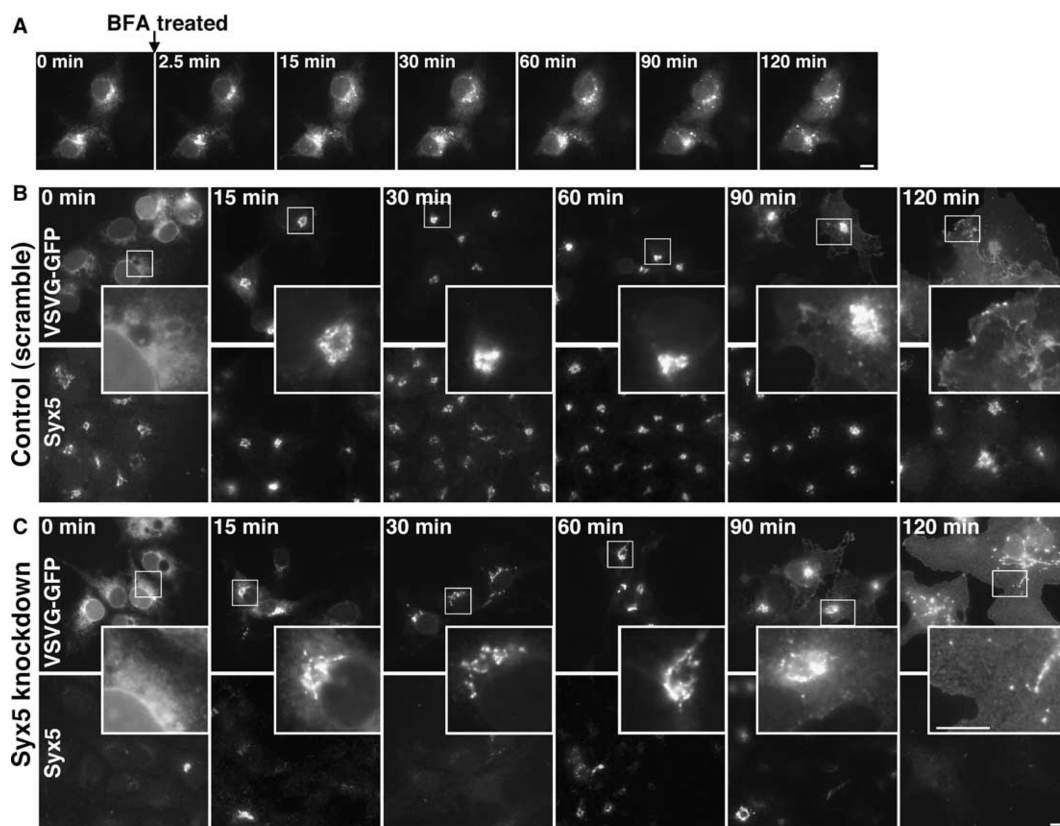

Fig. 4. Effect of Syx5 knockdown on vesicle trafficking. (A) COS-7 cells transfected with a VSVG-GFP expression plasmid were treated with BFA (at 2 min), and consecutive images were taken of the same cells. (B) COS-7 cells were transfected with the scrambled duplex siRNA followed by transfection with a VSVG-GFP-expressing plasmid. The transfected cells were maintained at 40 °C for 14–16 h, shifted to 32 °C, and incubated for the indicated times. After fixation at each time point, the cells were subjected to immunocytochemistry. (C) Cells were transfected with duplex siRNA of Syx5, and an experiment similar to that described in (B) was performed. Upon shifting to the permissive temperature, VSVG-GFP was transported from the ER to the Golgi apparatus in 30 min and was finally transported to the plasma membrane in 120 min both in control (B) and knockdown cells (C), whereas the transportation of VSVG-GFP along the secretory pathway was significantly inhibited in BFA-treated cells, as shown in (A). Insets show magnified views of boxed areas. Bars, 10 μm.

the accumulation of green fluorescent protein-tagged VSVG (VSVG-GFP) in the ER (Fig. 4A). Fragmentation of the Golgi was also detected in cells in which Syx5 was overexpressed (Fig. 3C); therefore, we also investigated the effects of Syx5 overexpression on the transport of VSVG-GFP. As was found in other cells [7], when Syx5 was overexpressed in HeLa cells, VSVG-GFP was predominantly retained in the ER at 30 min after the shift to the permissive temperature, while VSVG-GFP strictly localized at the Golgi apparatus in control cells (data not shown). In cells in which Syx5 was overexpressed, the retention of VSVG-GFP in the ER, ERGIC/VTC, and Golgi apparatus persisted for over 2 h after the temperature shift (data not shown). These results suggest that the excess Syx5 expression interferes with the anterograde transport of vesicles in the ER and Golgi compartments.

In a final series of experiments, COS-7 cells were transfected with siRNA followed by a plasmid expressing VSVG-GFP, and the transport was analyzed. In control cells transfected with scrambled siRNA, VSVG-GFP was localized in the ER at the nonpermissive temperature (time, 0 min); after shifting to the permissive temperature, VSVG-GFP was transported to the Golgi apparatus (time, 30 min) and finally to the plasma membrane at 120 min (Fig. 4B). To our surprise, in Syx5 knockdown cells, VSVG-GFP was efficiently transported from the ER to the plasma membrane (Fig. 4C). The population of cells of which plasma membrane were VSVG-GFP-positive at time 120 min were 83% and 85% in control and Syx5 knockdown cells, respectively. As can be seen in Fig. 4B and C, although VSVG-GFP was localized to the fragmented Golgi, VSVG-GFP was capable of transport along the secretory pathway in COS-7 cells. While, at 30 min, 90% of the VSVG-positive cells treated with scrambled siRNA showed VSVG localization in ribbon-like structures, 89% of the VSVG-positive cells treated with Syx5 siRNA showed VSVG localization in fragmented structures. These VSVG location were well co-localized with the GM130 positive Golgi fragments (data not shown). Similar results were obtained in HeLa cells transfected with either duplex siRNA or a plasmid expressing siRNA (data not shown). The transport of VSVG-GFP across the Golgi compartment was not diminished by the major decrease in Syx5 that is associated with Golgi fragmentation.

4. Discussion

In order to show the physiological significance of Syx5 in cellular function, we have generated a novel monoclonal antibody specific to Syx5 and have analyzed the Golgi structures and vesicle transport along the secretory pathway under two sets of conditions. One of these is the knockdown

of the expression of the Syx5 gene by an RNA interference method, and the other is Syx5 overexpression. Remarkably, we found that Syx5 depletion by RNAi results in extensive fragmentation of the Golgi apparatus (Fig. 3), supporting a role for Syx5 in the maintenance of Golgi architecture. In support of this idea, reassembly experiments of Golgi stacks in a cell-free system revealed that Syx5 is likely to be involved in the organization of Golgi cisternae at mitosis [12]. The absence of Syx5 may affect the biogenesis of the Golgi apparatus in cooperation with other vesicle transport regulator proteins or protein complexes that interact with Syx5. One such candidate is rSly1 [35], a member of the nSec-1/munc18 (SM) group of proteins, because it has been shown that blocking rSly1 binding to Syx5 results in the disruption of the Golgi apparatus [36]. In addition, studies in vitro showed that rSly1 binding to Syx5 is required for ER-to-Golgi transport [37]. Another candidate is the p115 protein, which tethers COPI vesicles to the Golgi membrane and is essential for the functioning of the Golgi apparatus [38]. It has also been demonstrated that the p115 protein directly interacts with Golgi SNAREs, including Syx5, and catalyzes SNARE complex assembly [39]. Other candidates are conserved oligomeric Golgi (COG) complexes, which are suggested to be necessary for the membrane trafficking modulated by COPI and were shown to interact with COPI and the Golgi SNARE GS28 [40]. The immunoreactivity of βCOP and GM130 co-localized in Syx5 knockdown cells (Fig. 3B, merged images), indicating that COPI vesicles might be capable of tethering to the fragmented Golgi. Furthermore, similar to the results seen in other cells [40], these fragmented Golgi might be competent for the anterograde transport of membrane proteins, at least for VSVG transport.

Although it is still not clear if COPs act directly or indirectly in the anterograde or retrograde direction in the ER, ERGIC/VTC, and Golgi compartment [41–43], VSVG protein can potentially enter COPI and COPII vesicles [31,44,45]. A number of reports have shown that Syx5 and VSVG are co-localized in the same COP-coated vesicles [7,13,46–48]. Furthermore, the distribution and combinatorial specificity of yeast and mammalian SNARE complexes containing Syx5 mediate the flow and the fusion of COP vesicles [10,18,21,49–54]. As all of these findings imply a significant role of Syx5 in intracellular vesicle transport, the lack of a marked effect of Syx5 knockdown on anterograde transport of VSVG was striking. Although some minor trafficking defects and/or kinetic delays in the transport of VSVG from the ER to the plasma membrane cannot be excluded, the finding that the majority of VSVG reaches the plasma membrane indicates that Syx5 may not be indispensable for the anterograde flow of some membrane proteins, at least VSVG-containing vesicles, along the secretory pathways. In mammalian cells, it is possible that undefined proteins, other than Syx5, may compensate for Syx5 in the transport of vesicles in the ER through Golgi compartments. Molecular redundancy of SNARE proteins in ER–Golgi transport has been reported in yeast cells [55]. Accordingly, further experiments in Syx5 knockdown cells examining whether other Syxs and/or SNAREs are upregulated and functionally substitute for Syx5 are required. It has been reported that Membrin, another Golgi SNARE that binds to Syx5, is involved in the targeting of ADP-ribosylation factor-1 (Arf-1) to the early phase of the Golgi [56]. It is noteworthy that the action of Membrin on the interaction and distribution of Arf-1 suggests an additional role of Membrin distinct from the function mediated by the SNARE complex. Our results also indicate the significance of Syx5 in cellular functions other than t-SNARE and raise the possibility that the SNARE hypothesis may have to be reconsidered. In future studies, by using Syx5 knockdown cells, precise investigation of the mechanism of Golgi fragmentation and vesicle transport mediated by SNARE complex formation is needed. In addition, it would be of interest to examine whether there is any defect in the retrograde transport of vesicles from the Golgi to the ER in Syx5 knockdown cells. A Syx5-ablated cell might be useful for clarifying the role of Syx5 and for the investigation of SNARE function as well as Syx5 functions distinct from SNARE.

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References

[1] Jahn, R., Lang, T. and Südhof, T.C. (2003) Membrane fusion. Cell 112, 519–533.

[2] Inoue, A., Obata, K. and Akagawa, K. (1992) Cloning and sequence analysis of cDNA for a neuronal cell membrane antigen, HPC-1. J. Biol. Chem. 267, 10613–10619.

[3] Inoue, A. and Akagawa, K. (1992) Neuron-specific antigen HPC-1 from bovine brain reveals strong homology to epimorphin, an essential factor involved in epithelial morphogenesis: identification of a novel protein family. Biochem. Biophys. Res. Commun. 187, 1144–1150.

[4] Bennett, M.K., Garcia-Arraras, J.E., Elferink, L.A., Peterson, K., Fleming, A.M., Hazuka, C.D. and Scheller, R.H. (1993) The syntaxin family of vesicular transport receptors. Cell 74, 863–873.

[5] Gaisano, H.Y., Ghai, M., Malkus, P.N., Sheu, L., Bouquillon, A., Bennett, M.K. and Trimble, W.S. (1996) Distinct cellular locations of the syntaxin family of proteins in rat pancreatic acinar cells. Mol. Biol. Cell 7, 2019–2027.

[6] Low, S.H., Chapin, S.J., Weimbs, T., Komuves, L.G., Bennett, M.K. and Mostov, K.E. (1996) Differential localization of syntaxin isoforms in polarized Madin–Darby canine kidney cells. Mol. Biol. Cell 7, 2007–2018.

[7] Dascher, C., Matteson, J. and Balch, W.E. (1994) Syntaxin 5 regulates endoplasmic reticulum to Golgi transport. J. Biol. Chem. 269, 29363–29366.

[8] Hay, J.C., Klumperman, J., Oorschot, V., Steegmaier, M., Kuo, C.S. and Scheller, R.H. (1998) Localization, dynamics, and protein interactions reveal distinct roles for ER and Golgi SNAREs. J. Cell Biol. 141, 1489–1502.

[9] Kasai, K. and Akagawa, K. (2001) Roles of the cytoplasmic and transmembrane domains of syntaxins in intracellular localization and trafficking. J. Cell Sci. 114, 3115–3124.

[10] Hui, N., Nakamura, N., Sönnichsen, B., Shima, D.T., Nilsson, T. and Warren, G. (1997) An isoform of the Golgi t-SNARE, syntaxin 5, with an endoplasmic reticulum retrieval signal. Mol. Biol. Cell 8, 1777–1787.

[11] Zhang, T., Wong, S.H., Tang, B.L., Xu, Y., Peter, F., Subramaniam, V.N. and Hong, W. (1997) The mammalian protein (rbet1) homologous to yeast Bet1p is primarily associated with the

pre-Golgi intermediate compartment and is involved in vesicular transport from the endoplasmic reticulum to the Golgi apparatus. J. Cell Biol. 139, 1157–1168.

[12] Rabouille, C., Kondo, H., Newman, R., Hui, N., Freemont, P. and Warren, G. (1998) Syntaxin 5 is a common component of the NSF- and p97-mediated reassembly pathways of Golgi cisternae from mitotic Golgi fragments in vitro. Cell 92, 603–610.

[13] Rowe, T., Dascher, C., Bannykh, S., Plutner, H. and Balch, W.E. (1998) Role of vesicle-associated syntaxin 5 in the assembly of pre-Golgi intermediates. Science 279, 696–700.

[14] Xu, D., Joglekar, A.P., Williams, A.L. and Hay, J.C. (2000) Subunit structure of a mammalian ER/Golgi SNARE complex. J. Biol. Chem. 275, 39631–39639.

[15] Roy, L. et al. (2000) Role of p97 and syntaxin 5 in the assembly of transitional endoplasmic reticulum. Mol. Biol. Cell 11, 2529–2542.

[16] Tsui, M.M., Tai, W.C. and Banfield, D.K. (2001) Selective formation of Sed5p-containing SNARE complexes is mediated by combinatorial binding interactions. Mol. Biol. Cell 12, 521–538.

[17] Zhang, T. and Hong, W. (2001) Ykt6 forms a SNARE complex with syntaxin 5, GS28, and Bet1 and participates in a late stage in endoplasmic reticulum–Golgi transport. J. Biol. Chem. 276, 27480–27487.

[18] Parlati, F., Varlamov, O., Paz, K., McNew, J.A., Hurtado, D., Söllner, T.H. and Rothman, J.E. (2002) Distinct SNARE complexes mediating membrane fusion in Golgi transport based on combinatorial specificity. Proc. Natl. Acad. Sci. USA 99, 5424–5429.

[19] Peng, R. and Gallwitz, D. (2002) Sly1 protein bound to Golgi syntaxin Sed5p allows assembly and contributes to specificity of SNARE fusion complexes. J. Cell Biol. 157, 645–655.

[20] Xu, Y., Martin, S., James, D.E. and Hong, W. (2002) GS15 forms a SNARE complex with syntaxin 5, GS28, and Ykt6 and is implicated in traffic in the early cisternae of the Golgi apparatus. Mol. Biol. Cell 13, 3493–3507.

[21] Volchuk, A. et al. (2004) Countercurrent distribution of two distinct SNARE complexes mediating transport within the Golgi stack. Mol. Biol. Cell 23, 23.

[22] Tai, G., Lu, L., Wang, T.L., Tang, B.L., Goud, B., Johannes, L. and Hong, W. (2004) Participation of the syntaxin 5/Ykt6/GS28/GS15 SNARE complex in transport from the early/recycling endosome to the TGN. Mol. Biol. Cell 15, 4011–4022.

[23] Glick, B.S. (2000) Organization of the Golgi apparatus. Curr. Opin. Cell. Biol. 12, 450–456.

[24] Lowe, M., Lane, J.D., Woodman, P.G. and Allan, V.J. (2004) Caspase-mediated cleavage of syntaxin 5 and giantin accompanies inhibition of secretory traffic during apoptosis. J. Cell Sci. 117, 1139–1150.

[25] Suga, K., Tomiyama, T., Mori, H. and Akagawa, K. (2004) Syntaxin 5 interacts with presenilin holoproteins, but not with their N- or C-terminal fragments, and affects β amyloid peptide production. Biochem. J. 381, 619–628.

[26] Suga, K., Saito, A., Tomiyama, T., Mori, H. and Akagawa, K. (2005) Syntaxin 5 interacts specifically with presenilin holoproteins and affects processing of βAPP in neuronal cells. J. Neurochem. 94, 425–439.

[27] Milhavet, O., Gary, D.S. and Mattson, M.P. (2003) RNA interference in biology and medicine. Pharmacol. Rev. 55, 629–648.

[28] Suga, K., Yamamori, T. and Akagawa, K. (2003) Identification of the carboxyl-terminal membrane-anchoring region of HPC-1/Syntaxin 1A with the substituted-cysteine-accessibility method and monoclonal antibodies. J. Biochem. (Tokyo) 133, 325–334.

[29] Duden, R., Griffiths, G., Frank, R., Argos, P. and Kreis, T.E. (1991) β-COP, a 110 kD protein associated with non-clathrin-coated vesicles and the Golgi complex, shows homology to β-adaptin. Cell 64, 649–665.

[30] Nakamura, N., Lowe, M., Levine, T.P., Rabouille, C. and Warren, G. (1997) The vesicle docking protein p115 binds GM130, a *cis*-Golgi matrix protein, in a mitotically regulated manner. Cell 89, 445–455.

[31] Presley, J.F., Cole, N.B., Schroer, T.A., Hirschberg, K., Zaal, K.J. and Lippincott-Schwartz, J. (1997) ER-to-Golgi transport visualized in living cells. Nature 389, 81–85.

[32] Hirschberg, K., Miller, C.M., Ellenberg, J., Presley, J.F., Siggia, E.D., Phair, R.D. and Lippincott-Schwartz, J. (1998) Kinetic analysis of secretory protein traffic and characterization of golgi to plasma membrane transport intermediates in living cells. J. Cell Biol. 143, 1485–1503.

[33] Doms, R.W., Russ, G. and Yewdell, J.W. (1989) Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. J. Cell Biol. 109, 61–72.

[34] Lippincott-Schwartz, J., Yuan, L.C., Bonifacino, J.S. and Klausner, R.D. (1989) Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell 56, 801–813.

[35] Dascher, C. and Balch, W.E. (1996) Mammalian Sly1 regulates syntaxin 5 function in endoplasmic reticulum to Golgi transport. J. Biol. Chem. 271, 15866–15869.

[36] Yamaguchi, T., Dulubova, I., Min, S.W., Chen, X., Rizo, J. and Südhof, T.C. (2002) Sly1 binds to Golgi and ER syntaxins via a conserved N-terminal peptide motif. Dev. Cell 2, 295–305.

[37] Williams, A.L., Ehm, S., Jacobson, N.C., Xu, D. and Hay, J.C. (2004) rsly1 binding to syntaxin 5 is required for ER to Golgi transport but does not promote SNARE motif accessibility. Mol. Biol. Cell 16, 162–175.

[38] Puthenveedu, M.A. and Linstedt, A.D. (2004) Gene replacement reveals that p115/SNARE interactions are essential for Golgi biogenesis. Proc. Natl. Acad. Sci. USA 101, 1253–1256.

[39] Shorter, J., Beard, M.B., Seemann, J., Dirac-Svejstrup, A.B. and Warren, G. (2002) Sequential tethering of Golgins and catalysis of SNAREpin assembly by the vesicle-tethering protein p115. J. Cell Biol. 157, 45–62.

[40] Zolov, S.N. and Lupashin, V.V. (2005) Cog3p depletion blocks vesicle-mediated Golgi retrograde trafficking in HeLa cells. J. Cell Biol. 168, 747–759.

[41] Klumperman, J. (2000) Transport between ER and Golgi. Curr. Opin. Cell Biol. 12, 445–449.

[42] Storrie, B., Pepperkok, R. and Nilsson, T. (2000) Breaking the COPI monopoly on Golgi recycling. Trends Cell Biol. 10, 385–391.

[43] Palmer, K.J. and Stephens, D.J. (2004) Biogenesis of ER-to-Golgi transport carriers: complex roles of COPII in ER export. Trends Cell Biol. 14, 57–61.

[44] Balch, W.E., McCaffery, J.M., Plutner, H. and Farquhar, M.G. (1994) Vesicular stomatitis virus glycoprotein is sorted and concentrated during export from the endoplasmic reticulum. Cell 76, 841–852.

[45] Scales, S.J., Pepperkok, R. and Kreis, T.E. (1997) Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. Cell 90, 1137–1148.

[46] Nishimura, N., Bannykh, S., Slabough, S., Matteson, J., Altschuler, Y., Hahn, K. and Balch, W.E. (1999) A di-acidic (DXE) code directs concentration of cargo during export from the endoplasmic reticulum. J. Biol. Chem. 274, 15937–15946.

[47] Orci, L. et al. (2000) Anterograde flow of cargo across the golgi stack potentially mediated via bidirectional "percolating" COPI vesicles. Proc. Natl. Acad. Sci. USA 97, 10400–10405.

[48] Xu, D. and Hay, J.C. (2004) Reconstitution of COPII vesicle fusion to generate a pre-Golgi intermediate compartment. J. Cell Biol. 167, 997–1003.

[49] Hardwick, K.G. and Pelham, H.R. (1992) SED5 encodes a 39-kD integral membrane protein required for vesicular transport between the ER and the Golgi complex. J. Cell Biol. 119, 513–521.

[50] Banfield, D.K., Lewis, M.J., Rabouille, C., Warren, G. and Pelham, H.R. (1994) Localization of Sed5, a putative vesicle targeting molecule, to the *cis*-Golgi network involves both its transmembrane and cytoplasmic domains. J. Cell Biol. 127, 357–371.

[51] Holthuis, J.C., Nichols, B.J., Dhruvakumar, S. and Pelham, H.R. (1998) Two syntaxin homologues in the TGN/endosomal system of yeast. EMBO J. 17, 113–126.

[52] Wooding, S. and Pelham, H.R. (1998) The dynamics of golgi protein traffic visualized in living yeast cells. Mol. Biol. Cell 9, 2667–2680.

[53] Miller, E.A., Beilharz, T.H., Malkus, P.N., Lee, M.C., Hamamoto, S., Orci, L. and Schekman, R. (2003) Multiple cargo binding sites on the COPII subunit Sec24p ensure capture of diverse membrane proteins into transport vesicles. Cell 114, 497–509.
[54] Mossessova, E., Bickford, L.C. and Goldberg, J. (2003) SNARE selectivity of the COPII coat. Cell 114, 483–495.
[55] Liu, Y. and Barlowe, C. (2002) Analysis of Sec22p in endoplasmic reticulum/Golgi transport reveals cellular redundancy in SNARE protein function. Mol. Biol. Cell 13, 3314–3324.
[56] Honda, A., Al-Awar, O.S., Hay, J.C. and Donaldson, J.G. (2005) Targeting of Arf-1 to the early Golgi by membrin, an ER–Golgi SNARE. J. Cell Biol. 168, 1039–1051.