Contribution of the long form of syntaxin 5 to the organization of the endoplasmic reticulum

Kaori Miyazaki*, Yuichi Wakana*, Chikano Noda*, Kohei Arasaki, Akiko Furuno and Mitsuo Tagaya‡

School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan

*These authors contributed equally to this work.
‡Author for correspondence (tagaya@ls.toyaku.ac.jp)

Accepted 20 September 2012
Journal of Cell Science 125, 5658–5666

doi: 10.1242/jcs.105304

Summary

The SNARE protein syntaxin 5 exists as long (42 kDa) and short (35 kDa) isoforms. The short form is principally localized in the Golgi complex, whereas the long form resides not only in the Golgi but also in the endoplasmic reticulum (ER). Although the Golgi-localized short form has been extensively investigated, little is known about the long form. In the present study, we demonstrate that the long form of syntaxin 5 functions to shape the ER. We found that overexpression of the long form of syntaxin 5 induces rearrangement and co-alignment of the ER membrane with microtubules, the pattern of which is quite similar to that observed in cells overexpressing CLIMP-63, a linker between the ER membrane and microtubules. The ability of syntaxin 5 to induce ER-microtubule rearrangement is not related to its SNARE function, but correlates with its binding affinities for CLIMP-63, and CLIMP-63 is essential for the induction of this rearrangement. Microtubule co-sedimentation assays demonstrated that the long form of syntaxin 5 has a substantial microtubule-binding activity. These results suggest that the long form of syntaxin 5 contributes to the regulation of ER structure by interacting with both CLIMP-63 and microtubules. Indeed, depletion of syntaxin 5 caused the spreading of the ER to the cell periphery, similar to the phenotype observed in cells treated with the microtubule-depolymerizing reagent nocodazole. Our results disclose a previously undescribed function of the long form of syntaxin 5 that is not related to its function as a SNARE.

Key words: CLIMP-63, Endoplasmic reticulum, Golgi, Microtubule, Syntaxin 5

Introduction

The endoplasmic reticulum (ER) is a continuous membrane structure composed of sheet-like cisternae and a polygonal array of tubules connected by three-way junctions (Baumann and Walz, 2001; English et al., 2009; Shibata et al., 2009; Pendin et al., 2011). The ER structure is highly dynamic; new membrane tubules are continuously formed and fused with other tubules to form three-way junctions (Lee and Chen, 1988). The ER has a variety of functions such as protein synthesis and export, lipid synthesis, and calcium storage and homeostasis. These functions are achieved by subdomains or contact sites with other organelles, both of which are formed in a continuous network through poorly understood mechanisms (English et al., 2009; Lynes and Simmern, 2011).

In animal cells, microtubules (MTs) play important roles in the organization and dynamics of ER tubules (Dabora and Sheetz, 1988; Lee et al., 1989; Terasaki and Reese, 1994; Vedrenne and Hauri, 2006). There are several mechanisms by which ER membranes interact with MTs. First, they bind to the growing tips of MT through tip attachment complexes (Waterman-Storer and Salmon, 1998). A recent study showed that an ER membrane protein, STIM1, and a MT plus end-binding protein, EB1, are responsible for this interaction (Grigoriev et al., 2008). Second, ER tubules initially bind to the shaft of an existing MT and slide along the MT (Waterman-Storer and Salmon, 1998; Lee et al., 1989). This sliding occurs predominantly on curved, acetylated MTs (Friedman et al., 2010) and is driven by MT motors, kinesin and cytoplasmic dynein (Wozniak and Allan, 2006; Wozniak et al., 2009). The third mechanism is a static interaction between ER membranes and MTs, which is mediated by some proteins (Vedrenne and Hauri, 2006).

Syntaxin 5 (Syn5) is a member of the soluble NSF attachment protein receptor (SNARE) family of proteins implicated in membrane fusion in vesicular transport processes (Hong, 2005; Jahn and Scheller, 2006). Syn5 regulates the transport from the ER to the Golgi (Dascher et al., 1994; Hay et al., 1997; Rowe et al., 1998; Bentley et al., 2006) and from the early/recycling endosomes to the *trans*-Golgi network (Tai et al., 2004; Amessou et al., 2007). It also participates in the assembly of transitional ER (Lavoie et al., 2000; Roy et al., 2000) and the Golgi (Rabouille et al., 1998; Müller et al., 2002; Suga et al., 2005), lipid droplet fusion (Boström et al., 2007), and cytokinesis (Xu et al., 2002).

Syn5 exists in two forms, a 42-kDa form [herein referred to as Syn5 (long form)] and a 35-kDa form [referred to as Syn5 (short form)] (Hui et al., 1997). The two isoforms are likely formed from a single mRNA through alternative initiation of translation, and Syn5 (long form) has an N-terminal cytoplasmic extension containing a predicted type II ER retrieval signal. Consistent with the presence of the ER retrieval motif, there is less of Syn5 (long form) in the Golgi complex compared with the short form (Hui et al., 1997). Although the role of Syn5 in the Golgi has been substantially investigated, little is known about the function of Syn5 (long form). There is only one report showing that the two forms may have different effects on β-amyloid processing (Suga et al., 2009).

In this study we reveal an unexpected link between Syn5 and MTs. We show that Syn5 contributes to the organization of the

ER structure by interacting with MTs and CLIMP-63, an ER integral membrane protein that regulates the ER structure through a static interaction with MTs (Klopfenstein et al., 1998) as well as through acting as a 'luminal ER spacer' (Shibata et al., 2010).

Results

Overexpression of Syn5 (long form) induces rearrangement of the ER network

Like other syntaxin molecules, both Syn5 (long form) and Syn5 (short form) contain an N-terminal domain consisting of three helices (Ha, Hb, Hc) and a C-terminal SNARE motif followed by a transmembrane domain. The former species possesses a double-arginine motif (RKR) at its extended N-terminal region (Fig. 1A). Although the double-arginine motif in the N-terminal extension of Syn5 (long form), when grafted to the 31-kDa form of major histocompatibility complex class II invariant chain, was found to function as an ER retrieval signal, the distribution of expressed Syn5 (long form) was not precisely investigated (Hui et al., 1997). We therefore first sought to determine the localization of Syn5 (long form) with a FLAG tag expressed in COS-7 cells. Unexpectedly, FLAG-Syn5 (long form) exhibited a tubular pattern surrounding the nucleus in many cells (Fig. 1B, top two rows). Double staining for the expressed protein and an ER marker, HSP47 (Fig. 1B, top row), or α-tubulin (second row) revealed that this pattern reflects rearrangement of the ER network accompanied by MT reorganization. In contrast to Syn5 (long form), FLAG-Syn5 (short form), like endogenous protein, was co-localized with a Golgi marker, GM130, in many cells (Fig. 1B, third row). The ability of Syn5 (long form) to induce ER-MT rearrangement is independent of its membrane fusion activity, because this rearrangement also occurred when Syn5Δ269-338, which lacks the C-terminal SNARE motif, was overexpressed (Fig. 1B, bottom row).

Interaction of Syn5 (long form) with CLIMP-63

As the localization pattern of overexpressed Syn5 (long form) was quite similar to that of overexpressed CLIMP-63 (Klopfenstein et al., 1998), we compared the patterns of the two proteins. As reported previously (Klopfenstein et al., 1998), overexpressed CLIMP-63 caused the rearrangement of the ER co-aligning with MTs (supplementary material Fig. S1, top two rows). In Syn5 (long form)-expressing cells, CLIMP-63 was co-localized with FLAG-Syn5 (long form) (bottom two rows).

Given that the phenotypes of cells expressing Syn5 (long form) and CLIMP-63 were essentially the same, we investigated whether or not the two proteins interact with each other. As

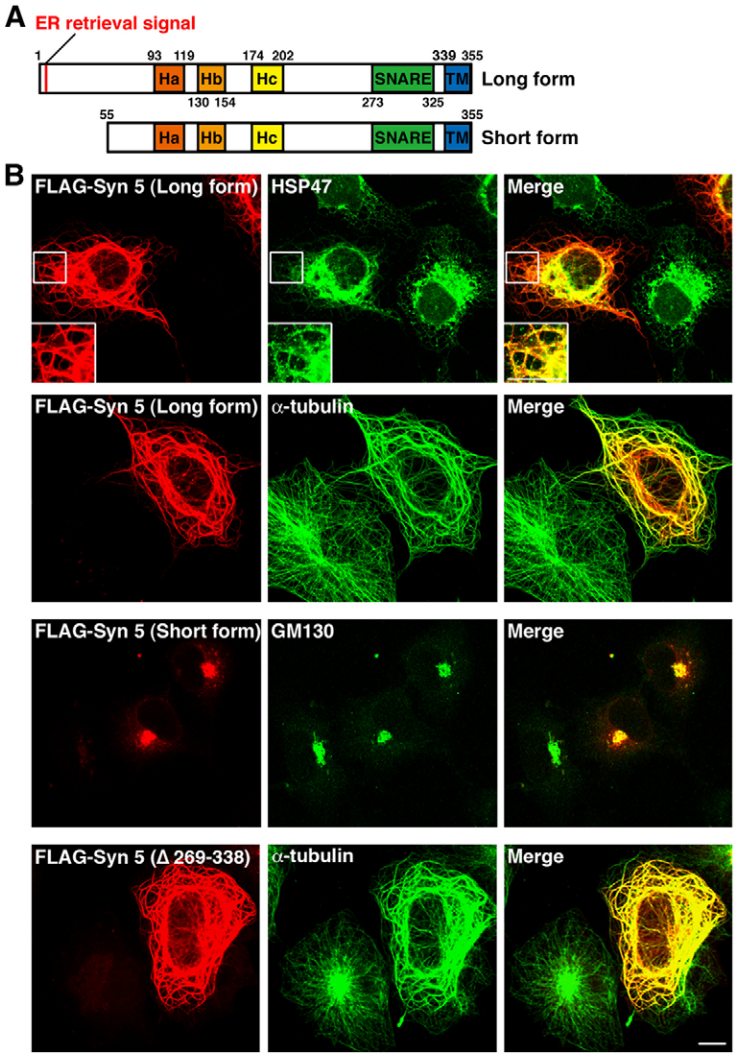


Fig. 1. The two expressed Syn5 isoforms exhibit different cellular localization. (**A**) Representation of two Syn5 isoforms, a 42-kDa long form and a 35-kDa short form. The former has a longer N-terminal region containing an ER retrieval signal. Both isoforms possess a three-helical bundle (consisting of Ha, Hb and Hc regions), a SNARE motif and a transmembrane (TM) domain. (**B**) The expressed long form of Syn5 displays a filamentous distribution and is co-localized with the ER. At 18 hours after transfection with the plasmid for each of the FLAG-Syn5 constructs, COS-7 cells were fixed and double-stained for FLAG and HSP47 (top row), α-tubulin (second and bottom rows) or GM130 (third row). Syn5 (Δ269-338) indicates the long form of Syn5 lacking amino acids 269-338. The boxed areas are enlarged in the insets. Scale bars: 10 μm.

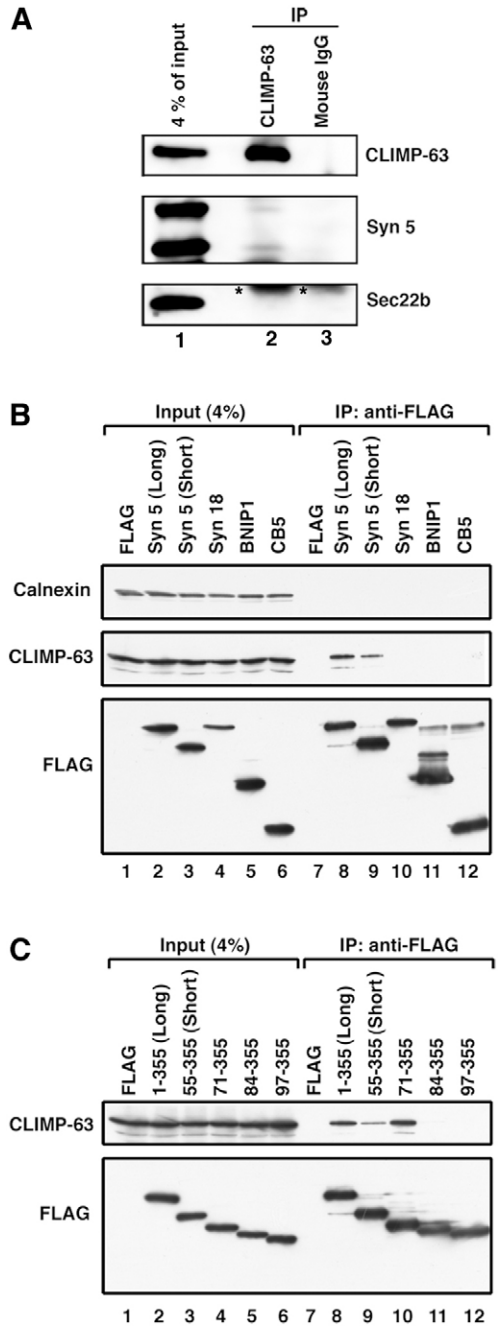


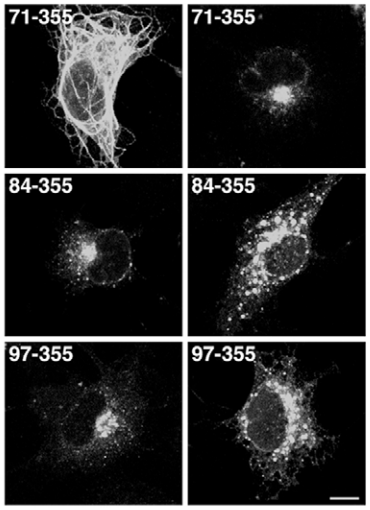
Fig. 2. Syn5 associates with CLIMP-63. (**A**) Membrane fractions of 293T cell lysates were prepared, solubilized and immunoprecipitated with an anti-CLIMP 63 antibody (lane 2) or a control IgG (lane 3). The precipitates were separated by SDS-PAGE and analyzed with the indicated antibodies. Asterisks indicate IgG light chain. The experiment was repeated with similar results. (**B**,**C**) Lysates of 293T cells expressing FLAG constructs including the long form (Long) and short form (Short) of Syn5 were subjected to immunoprecipitation with anti-FLAG M2 beads and immunoblotted with the indicated antibodies.

shown in Fig. 2A, a small but significant amount of endogenous Syn5 was co-precipitated with a monoclonal anti-CLIMP-63 antibody (middle panel, lane 2), whereas no co-precipitation of Syn 5 was observed with control mouse IgG (lane 3). Having demonstrated the interaction of endogenous Syn5 and CLIMP-63, we next examined whether Syn5 specifically binds to CLIMP-63. As shown in Fig. 2B, CLIMP-63 was co-precipitated with both FLAG-Syn5 (long form) (middle panel, lane 8) and FLAG-Syn5 (short form) (lane 9), although it was more efficiently co-precipitated with the former than with the latter. No co-precipitation was observed between Syn5 (long form) and calnexin (top panel, lane 8), suggesting that the binding is specific. The specificity of the interaction between CLIMP-63 and Syn5 (long form) was further demonstrated by the result that CLIMP-63 was not co-precipitated with any of the other type II ER membranes proteins tested [syntaxin 18 (Syn18), BNIP1, and cytochorme b_5 (CB5)] tagged with FLAG (Fig. 2B, middle panel, lanes 10-12).

To define the region of Syn5 responsible for the interaction with CLIMP-63, we constructed several N-terminally truncated Syn5 mutants and performed immunoprecipitation experiments. As shown in Fig. 2C, CLIMP-63 was not co-precipitated with Syn5 (amino acids 84-355) (upper panel, lane 11) or Syn5 (amino acids 97-355) (lane 12), but co-precipitated with Syn5 (amino acids 71-355) (lane 10), implying that amino acids 71-83 is important for the binding with CLIMP-63. Notably, CLIMP-63 was more effectively co-precipitated with Syn5 (amino acids 71-355) than with Syn5 (short form; amino acids 55-355). This may suggest that amino acids 55-70 interfere with the interaction with CLIMP-63. Although the precise mechanism for the different binding affinities of Syn5 (short form) and Syn5 (amino acids 71-355) for CLIMP-63 is not clear, the latter mutant might be very useful to test the correlation between CLIMP-63-binding ability and rearrangement of the ER structure. As shown in Fig. 3, FLAG-Syn5 (amino acids 71-355) exhibited two patterns; filamentous (top, left panel) and Golgi-like juxtanuclear (top, right panel) distributions. Quantification (Fig. 3, panel at the bottom) shows that the ability of Syn5 (amino acids 71-355) to induce filamentous structures was comparable to that of Syn5 (long form). On the other hand, Syn5 (amino acids 84-355) and Syn5 (amino acids 97-355) mainly displayed a juxtanuclear accumulation or a peripheral patchy distribution (Fig. 3, middle and bottom rows). They showed a filamentous distribution in only 15% of the cells. These results strongly suggest that amino acids 71-83 of Syn5 are important for both the interaction with CLIMP-63 and the induction of ER reorganization.

In some proteins including MT-associated proteins, basic amino acid residues are responsible for protein-protein interactions (Ye and Sloboda, 1997). There are two basic amino acids (Arg-74 and Lys-82) in the region of amino acids 71-83 (L^{71}QTRQNGIQTNKP^{83}). Therefore, these residues were individually replaced with Ala, and the ability of the resultant mutants to bind to CLIMP-63 was examined by immunoprecipitation. As shown in supplementary material Fig. S2A, the amounts of CLIMP-63 co-precipitated with these mutants, especially the K82A mutant, were substantially low (upper panel, lanes 5 and 6) compared to that co-precipitated with wild-type CLIMP-63 (lane 4). In parallel with the decreased binding affinities for CLIMP-63, these mutants exhibited decreased activities to induce ER-MT rearrangement (supplementary material Fig. S2B).

CLIMP-63 has a single transmembrane domain with the long C-terminal region in the lumen of the ER and the N-terminus facing the cytoplasm. Klopfenstein et al. (Klopfenstein et al., 1998) showed that amino acids 2-21 and 24-101 in the cytoplasmic region are an ER rearrangement determinant and a MT-binding region, respectively. Given that overexpression of



Constructs	Filamentous structures	Juxtanuclear accumulation	Peripheral patches	Other
Long form (aa 1-355)	63.3%	29.8%	5.0%	1.9%
Short form (aa 55-355)	20.1	53.6	24.0	2.2
Mutant (aa 71-355)	50.9	36.5	8.4	4.2
Mutant (aa 84-355)	14.8	42.7	38.0	4.4
Mutant (aa 97-355)	15.9	40.3	39.3	4.5

Fig. 3. Distribution patterns of truncation mutants of Syn5. At 18 hours after transfection with one of the plasmids for FLAG-tagged mutants of Syn5, COS-7 cells were fixed and stained for FLAG. Filamentous structures (top left), juxtanuclear accumulation (top right, middle left and bottom left) and peripheral patches (middle right and bottom right). Quantification data (average values of two independent experiments) are shown below. In each experiment, about 100 cells expressing FLAG-tagged constructs were analyzed. Scale bar: 10 μm.

Syn5 (long form) causes ER rearrangement, we wondered if the ER rearrangement determinant in CLIMP-63 is involved in the association with Syn5 (long form). To examine this possibility, we coexpressed FLAG-Syn5 (long form) with wild-type CLIMP-63 or a mutant, which lacks amino acids 2-21, and performed immunoprecipitation. As shown in supplementary material Fig. S2C, the amount of CLIMP-63 lacking the ER rearrangement determinant (CLIMP-63Δ2-21) co-precipitated with FLAG-Syn5 (long form) (upper panel, lane 6) was much lower than that of wild-type CLIMP-63 (lane 5), implying a link of the ER rearrangement ability of CLIMP-63 with its binding to Syn5.

Syn5 (long form) interacts with MTs

Next, we examined whether or not Syn5 interacts with polymerized MTs. To this end, transmembrane domain-deleted Syn5 proteins fused to the C-terminus of maltose-binding protein (MBP) were expressed in *Escherichia coli* and purified. The purified proteins were mixed with taxol-stabilized MTs, and then subjected to sedimentation as described under Materials and Methods. As shown in Fig. 4A, MBP-Syn5 (long form) (upper panel, lane 6), but not MBP-Syn5 (short form) (lane 9) or MBP (lane 3), co-sedimented with polymerized MTs. The K82A mutant, which exhibited a markedly reduced activity to induce ER-MT rearrangement (supplementary material Fig. S2B), was

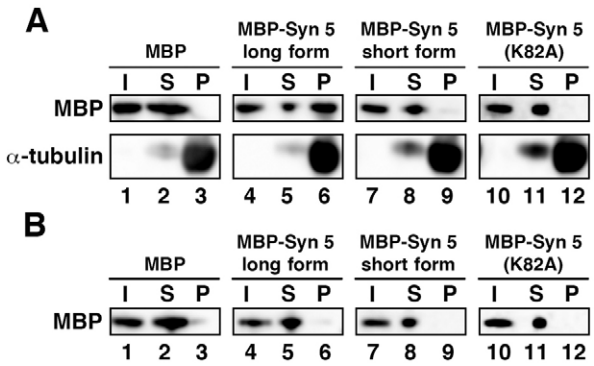


Fig. 4. The long form of Syn5 binds to polymerized MTs. (**A**,**B**) Purified MBP (lanes 1-3), MBP-Syn5 (long form) (lanes 4-6), MBP-Syn5 (short form) (lanes 7-9) and MBP-Syn5 (long form, K82A) (lanes 10-12), all lacking the transmembrane domain, were subjected to sedimentation with (A) or without (B) taxol-stabilized MTs, as described in Materials and Methods. Note that MBP-Syn5 (long form) was precipitated in the presence of taxol-stabilized MTs (A, lane 6), but not in their absence (B, lane 6), indicating the specificity of the assay. I, input; S, supernatant; P, precipitate.

not co-precipitated with polymerized MTs (Fig. 4A, lane 12). These results suggest that Syn5 has an ability to bind to polymerized MTs, and that its binding site for MTs is likely the same as that for CLIMP-63.

Loss of Syn5 induces ER spreading

To substantiate that Syn5 plays a role in shaping the ER, we treated HeLa cells with short interfering RNA (siRNA) targeting Syn5, and observed the distribution of ER proteins. As shown in supplementary material Fig. S3A, two siRNA effectively depressed the expression of Syn5; 22% of the control level in siRNA (432)-treated cells and 9% in siRNA (390)-treated cells. In parallel with the levels of Syn5 expression, the Golgi marker GM130 showed marginally and fully dispersed patterns in siRNA (432)- and siRNA (390)-treated cells, respectively (supplementary material Fig. S3B). Depletion of Syn5 did not affect the distribution of α-tubulin or acetylated tubulin (supplementary material Fig. S4).

Although siRNA (432) and siRNA (390) had considerably different effects on the Golgi structure, they similarly caused the redistribution of the ER, as visualized by staining for Sec61β (Fig. 5A, right) and CLIMP-63 (Fig. 5B, right). Quantification showed a marked decrease of the fluorescence intensity of Sec61β staining in the perinuclear region accompanied by some increase in regions distant from the nucleus (Fig. 5A, bottom panel). These results suggest that the ER structure was reorganized by the depletion of Syn5. An extension of CLIMP-63 staining to the cell periphery was also observed in COS-7 cells depleted of Syn5 (supplementary material Fig. S5). This ER alteration was not caused by depletion of Sec22b, a cognate partner of Syn5 (supplementary material Fig. S6). Notably, the Golgi complex, visualized by GM130 staining, was fragmented in Sec22b-depleted cells. In cells treated with Syn5 (432), on the other hand, ER spreading occurred without substantial Golgi fragmentation (Fig. 5A,B, middle row). These results suggest that ER reorganization induced by Syn5 depletion may not be attributable to the blocking of membrane trafficking between the ER and Golgi, although it remains possible that Syn5 knockdown

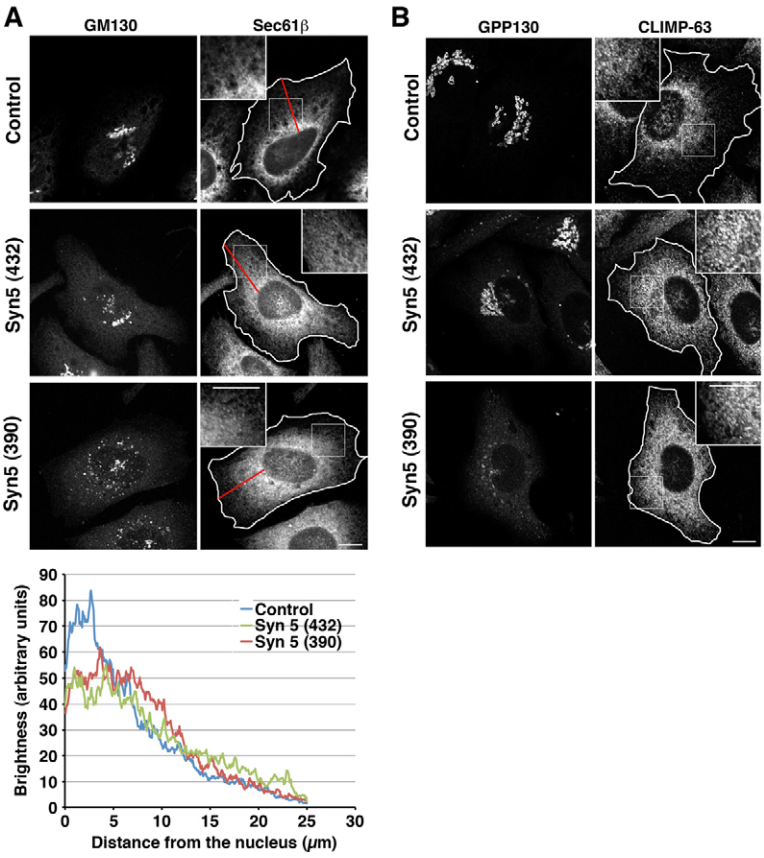


Fig. 5. Knockdown of Syn5 affects the sheet structure of the ER. (**A**,**B**) HeLa cells were transfected without (Control) or with one of the siRNAs targeting Syn5 [siRNA (432) or (390)]. At 72 hours after transfection, the cells were fixed and double-stained with antibodies against the Golgi marker GM130 and Sec61β (A) or the Golgi marker GPP130 and CLIMP-63 (B). The boxed areas are enlarged in the insets. White lines indicate cell edges. Sec61β fluorescence along a line (shown in red) from the nucleus to the cell periphery was analyzed using ImageJ software. The graph shows the distribution of Sec61β. The average values of fluorescence brightness in five cells are shown. Because the fluorescence intensity of CLIMP-63 changed rather irregularly, it was difficult to quantify CLIMP-63 distribution. Scale bars: 10 μm.

might result in vesicular trafficking defects that alter the ER structure.

ER distribution in Syn5-deficient cells is similar to that in nocodazole-treated cells

Given that Syn5 interacts with CLIMP-63 and MTs, we reasoned that ER extension to the cell periphery in Syn5-depleted cells is a consequence of the abrogation of the link between ER membranes and MTs. To explore this possibility, we treated cells with the MT-depolymerizing reagent nocodazole. In control cells, nocodazole treatment caused the redistribution of the centrally accumulated mRFP-Sec61β to more peripheral regions with the formation of lamellar/sheet-like structures at the cell periphery (Fig. 6, upper row, arrows; supplementary material Movie 1). In Syn5-depleted cells, on the other hand, mRFP-Sec61β exhibited an extended pattern without nocodazole treatment (Fig. 6, lower row, 0 min), although its pattern was not exactly the same as that observed in nocodazole-treated cells. Nocodazole treatment did not substantially affect the distribution of mRFP-Sec61β in Syn5-depleted cells (Fig. 6, lower row; supplementary material Movie 2). These results clearly demonstrated that the interaction of the ER with MTs is impaired in Syn5-deficient cells.

Syn5 silencing affects CLIMP-63-induced structure

We next examined whether depletion of Syn5 affects the distribution of expressed CLIMP-63. As reported previously (Klopfenstein et al., 1998) and also shown in this study, overexpression of CLIMP-63 induced MT bundling and reorganized the ER. If Syn5 cooperates with CLIMP-63 to regulate the ER structure, knockdown of Syn5 may affect the structure induced by the overexpression of CLIMP-63. In control cells, expressed CLIMP-63-FLAG exhibited tubular structures with several branches extending from the center to the cell periphery (Fig. 7A, upper left panel). We referred to this pattern as ‘bundles’. In Syn5-depleted cells, on the other hand, bundles were often observed as interconnected curvilinear structures with less branches (referred to as ‘circular bundles’) (Fig. 7A, lower panels).

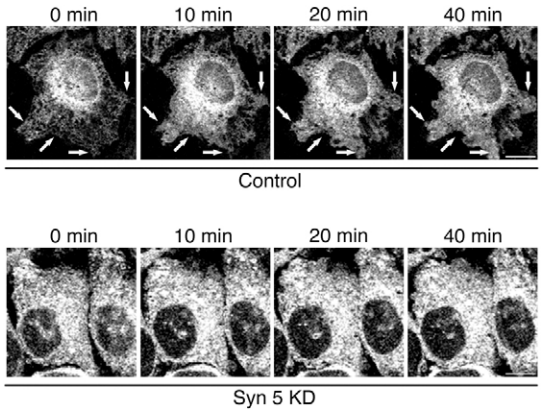


Fig. 6. ER structure in Syn5-silenced cells is similar to that in nocodazole-treated cells. At 72 hours after transfection of cells without (Control) or with siRNA (390) (Syn5 KD), cells stably expressing mRFP-Sec61β were incubated with 5 μg/ml nocodazole and live-cell imaging was performed at the indicated times. Arrows indicate peripheral cell regions, where lamellar/sheet-like ER structures were rarely seen before nocodazole treatment. Scale bars: 10 μm.

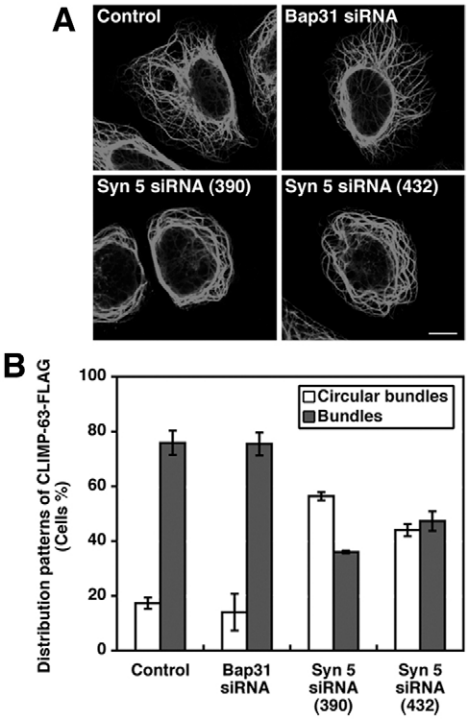


Fig. 7. Expressed CLIMP-63 forms circular bundles upon knockdown of Syn5. (**A**) At 48 hours after transfection without (Control) or with one of the siRNAs targeting Syn5 [siRNA (432) or (390)] or Bap31 (negative control), HeLa cells were transfected with the plasmid for CLIMP-63-FLAG and incubated for 18 hours. The cells were fixed and stained for FLAG. Scale bar: 10 μm. (**B**) Quantification of the distribution patterns of CLIMP-63-FLAG. In each experiment, about 100 cells expressing CLIMP 63-FLAG were analyzed. The average values with s.d. of three independent experiments are shown.

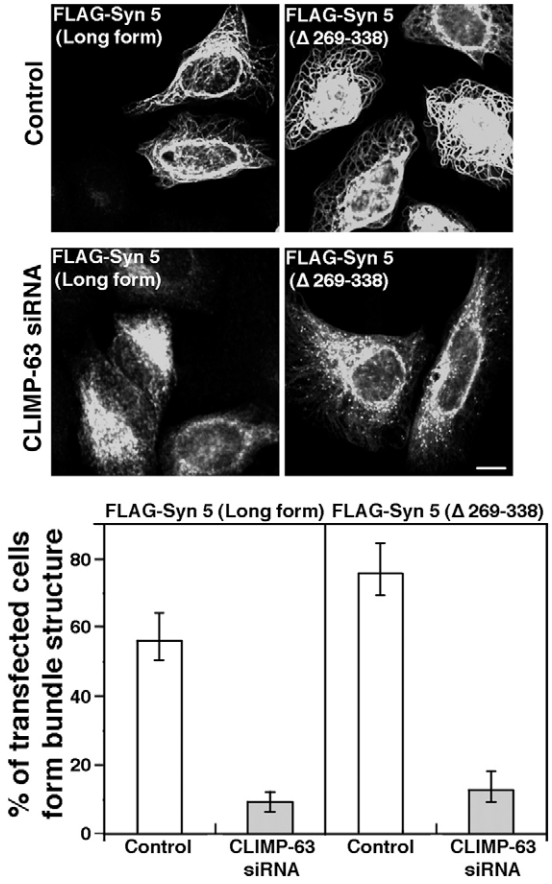


Fig. 8. CLIMP-63 is necessary for the reorganization of the ER induced by the long form of Syn5. (**A**) HeLa cells were transfected without (Control) or with siRNA targeting CLIMP-63. At 48 hours after transfection, the cells were transfected with the plasmid for FLAG-Syn5 (long form) or FLAG-Syn5 (Δ269-338). After 24 hours, the cells were fixed and stained for FLAG. Scale bar: 10 μm. (**B**) Quantification of the formation of the bundle structure induced by Syn5 constructs. In each experiment, about 100 Syn5-expressing cells were analyzed. The average values with s.d. of three independent experiments are shown.

It should be noted that the formation of circular bundles, as in the case of ER spreading (Fig. 5), was induced by siRNA (390) and siRNA (432) with nearly equal efficiency (Fig. 7B).

CLIMP-63 is necessary for the reorganization of the ER induced by Syn5 (long form)

To further demonstrate the link between Syn5 and CLIMP-63 in shaping the ER, we examined whether CLIMP-63 knockdown affects ER-MT rearrangement induced by the overexpression of Syn5 (long form). First, we investigated the ER structure in CLIMP-63-depleted cells. As shown in supplementary material Fig. S7A, CLIMP-63 was effectively knocked down, and as in the case of Syn5 knockdown, CLIMP-63 knockdown caused ER spreading as seen by Sec61β immunofluorescence (supplementary material Fig. S7B), in accordance with a previous result of Shibata et al. (Shibata et al., 2010). Nocodazole treatment caused a marked change in the distribution of Sec61β in control cells (supplementary material Fig. S7C, third versus top), whereas essentially no change was observed in CLIMP-63-depleted cells (supplementary material Fig. S7C, bottom versus second). As shown in Fig. 8, depletion of CLIMP-63 abrogated the ability of Syn5 (long form) to bundle the ER. Similar results were obtained for the Syn5 mutant lacking the SNARE domain (Syn5Δ269-338). The facts that CLIMP-63 is required for Syn5-induced ER reorganization (Fig. 8), whereas Syn5 is principally dispensable for CLIMP-63-induced ER reorganization (Fig. 7) suggest that CLIMP-63 and Syn5 play major and supportive roles, respectively, in the interaction of the ER with MTs.

Discussion

In this study we revealed that Syn5 (long form) functions to shape the ER. Overexpression of Syn5 (long form) caused the rearrangement of both the ER and MTs, leading to the formation of bundles. This rearrangement is not related to the membrane fusion activity of Syn5 because ER-MT rearrangement was also induced by a mutant lacking the SNARE domain. Depletion of Syn5 resulted in the spreading of the ER membrane toward the cell periphery. This extended pattern was reminiscent of that observed in cells treated with the MT-depolymerizing reagent nocodazole. Indeed, nocodazole treatment of Syn5-depleted cells did not substantially affect the ER structure, whereas control cells, when treated with nocodazole, exhibited a spreading ER morphology compared with that in nontreated cells. Immunoprecipitation and

sedimentation experiments demonstrated that Syn5 binds to both CLIMP-63, a MT-binding protein (Klopfenstein et al., 1998; Klopfenstein et al., 2001), and polymerized MTs. It is likely that the CLIMP-63-binding site on Syn5 is also responsible for the binding to polymerized MTs. These results taken together suggest that Syn5 (long form) contributes MT-binding activity that cooperates with that of CLIMP-63 to shape the ER.

MTs play an important role in the organization of the ER (Vedrenne and Hauri, 2006). Although depolymerization of MTs causes marked changes in the ER structure, the changes differ from one cell type to another. In newt lung epithelial cells and some cell types, slow retraction of the ER and clustering around the nucleus occur by nocodazole treatment (Lee et al., 1989; Terasaki et al., 1986; Terasaki and Reese, 1994; Waterman-Storer and Salmon, 1998). In Vero cells (Wozniak et al., 2009) and BSC1 cells (Lu et al., 2009), retraction of the ER is rare, and the ER undergoes a shift from reticular to lamellar/sheet-like morphology, with peripheral lamellar/sheet-like patches appearing. A similar conversion occurs during mitosis (Lu et al., 2009) perhaps because the extended network of MTs depolymerizes at the onset of mitosis, and the recovery of MTs does not take place until telophase (Zhai et al., 1996). Depletion of Syn5 from HeLa cells induced the extension of the ER, with the accumulation of lamellar/sheet-like structures at the cell periphery. The slow ER movement observed (supplementary material Movie S2) may be mediated by actomyosin. It should be noted that depletion of Syn5 also influenced the ER membrane structure induced by the overexpression of CLIMP-63 (Fig. 7). The circular bundle ER structure, albeit being formed as a result of overexpression artifacts, is similar to that observed in mitosis (Lu et al., 2009). Thus, this structure may be generated due to a Syn5 depletion-induced defect in the interaction of the ER with MTs.

Recent studies demonstrated that several families of proteins, such as the reticulon, DP1/Yop1 and atlastin families (Voeltz et al., 2006; Hu et al., 2009; Orso et al., 2009), are responsible for shaping the ER. These family proteins and the MT-severing protein spastin interact with one another and coordinate MT interactions with the tubular ER network (Park et al., 2010). Interestingly, overexpression of the MT-binding protein Reep1, a member of the DP1/YOP1 superfamily, has been shown to induce ER-MT rearrangement, as observed in cells overexpressing CLIMP-63 (Klopfenstein et al., 1998) and Syn5 (this study). Given that CLIMP-63 does not bind to atlastin-1 and is predominantly localized in the rough ER (Klopfenstein et al., 1998; Klopfenstein et al., 2001; Shibata et al., 2010), Park et al. (Park et al., 2010) point out the possibility that the Reep1 and CLIMP-63 play similar roles in mediating ER-MT interactions in different ER subdomains. As CLIMP-63 and Syn5 interact with each other, and both bind MTs, it is tempting to speculate that multivalent and cooperative interactions between ER membrane proteins and MTs are required for the organization of the rough ER, as in the case of the organization of the tubular ER network (Park et al., 2010). In this context, it is interesting that the overexpression of the C-terminal region of polycystin 1 causes ER-MT rearrangement (Gao et al., 2009). Polycystin 1 is known to form a heterodimer with polycystin 2 (Qian et al., 1997; Tsiokas et al., 1997), which has been reported to interact with Syn5 (Geng et al., 2008). Future studies should identify MT-binding proteins that interact with CLIMP-63 and Syn5.

The phenotype of Syn5-deficient cells is remarkably different from that of cells depleted of components of the Syn18 complex (Hirose et al., 2004). Depletion of Syn18 causes remarkable aggregation of the smooth ER membrane, although rough ER membranes are relatively intact (Iinuma et al., 2009). Knockdown of BNIP1 resulted in the loss of the three-way junction of the peripheral ER (Nakajima et al., 2004). These morphological changes may be ascribable to the inhibition of homotypic ER membrane fusion. On the other hand, depletion of Syn5 seems not to severely affect the fusion of ER membranes because no conspicuous morphological changes in the ER membrane were observed except for its extension to the cell periphery and reformation from tubular structures to lamellar/sheet-like ones. This is somewhat surprising because previous studies showed that VCP/p97 (Cdc48p in yeast) and its binding proteins mediate ER membrane fusion (Latterich et al., 1995; Uchiyama et al., 2002), and that Syn5 is a membrane receptor for VCP/p97 (Rabouille et al., 1998). It is possible that there are other VCP/p97 receptors in the ER that mediate ER network formation. Therefore, loss of Syn5 (long form) may not markedly affect the activity of VCP/p97 for ER network formation.

In yeast there is one Syn5 ortholog, Sed5 (Hardwick and Pelham, 1992; Banfield et al., 1994), which corresponds to the short form of Syn5. This may be related to the fact that the ER structure in yeast is shaped by actin, not MTs (Prinz et al., 2000; Du et al., 2004; West et al., 2011). To fulfill the requirement of MTs to shape the ER structure in mammalian cells, Syn5 (long form) might have been produced by an alternative initiation in the course of evolution. We do not exclude the possibility that the Syn5-mediated interaction with MTs may also contribute to the MT-dependent membrane trafficking from the ER to the Golgi complex. Of note, Syn5 (short form) also interacts, albeit relatively weakly, with CLIMP-63 (Fig. 2). This may suggest that Syn5 (short form) not only mediates membrane fusion in the Golgi, but also serves as a linker between MTs and transport vesicles destined for the Golgi complex.

In conclusion, our study demonstrated for the first time that Syn5 contributes to the organization of the ER through the interactions with MTs and CLIMP-63. This function is not related to the membrane fusion activity of Syn5.

Materials and Methods

Antibodies and reagents

A monoclonal antibody against HSP47 was purchased from Enzo Life Science. Monoclonal antibodies against GM130 and calnexin were from BD Biosciences Pharmingen. Monoclonal and polyclonal antibodies against FLAG and α-tubulin and a monoclonal antibody against acetylated tubulin were obtained from Sigma-Aldrich. Antiserum against Sec61β was purchased from Millipore Corp. Monoclonal antibodies against ERGIC-53 and CLIMP-63 were generous gifts from Dr H. P. Hauri. A polyclonal antibody against GPP130 was purchased from Covance. A polyclonal antibody against Syn5 was prepared in our laboratory (Mizoguchi et al., 2000). Nocodazole was obtained from Sigma-Aldrich.

Cell culture

COS-7 and 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin and 10% fetal calf serum. HeLa cells were cultured in Eagle's minimum essential medium supplemented with the same materials. To establish HeLa cells stably expressing mRFP-Sec61β, cells were transfected with the plasmid encoding mRFP-Sec61β and cultured in the presence of 400 μg/ml G418.

Plasmids and transfection

The plasmids encoding mRFP-Sec61β and CLIMP-63 were gifts from Dr T. Rapoport and H. P. Hauri, respectively. The cDNAs encoding wild-type Syn5, its deletion and N-terminally truncated mutants, full-length Syn18, full-length BNIP1, and Cytochrome b_5 (CB5) were inserted into pFLAG-CMV-6 (Sigma-Aldrich) so as to express proteins with an N-terminal FLAG tag. Ala substitutions of Arg-74 and Lys-82 in Syn5 were performed using PCR-based site-directed mutagenesis using the plasmid encoding FLAG-Syn5 (long form) as the template. To express Syn5 proteins lacking the transmembrane domain as MBP fusion proteins, the cDNAs

encoding Syn5 and its mutant were inserted into the *Bam*HI/*Hind*III site of pMal-C2X (New England Biolabs). Fusion proteins were expressed in *E. coli* and purified on amylose resin (New England Biolabs). The plasmid encoding CLIMP-63 with a C-terminal FLAG tag was constructed by inserting the CLIMP-63 cDNA to pFLAG-CMV-5 (Sigma-Aldrich). Transfection was carried out using LipofectAMINE PLUS (Invitrogen) according to the manufacturer's protocol.

Immunofluorescence microscopy

For immunofluorescence microscopy, cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature and observed with an Olympus Fluoview 300 or 1000 laser scanning microscope, as described previously (Tagaya et al., 1996).

Immunoprecipitation

For immunoprecipitation of endogenous CLIMP-63, ~90% confluent 293T cells (two 15-cm dishes) were washed twice in PBS and then once in homogenization buffer (20 mM Hepes-KOH (pH 7.2), 150 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μM pepstatin A, 2 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride). The cells were collected, suspended in 1 ml of homogenization buffer and homogenized with 20 strokes in a Dounce homogenizer. The homogenate was centrifuged at 1000 ***g*** for 10 minutes, and then the supernatant was centrifuged at 100,000 ***g*** for 30 minutes to separate the cytosol and membrane fractions. The membrane pellet was solubilized in homogenization buffer containing 1% Triton X-100. Equal volumes of Triton X-100 extracts were incubated for 1 hour with 2 μg of a monoclonal antibody against CLIMP-63 or a control mouse IgG. After incubation, 10 μl of protein G-Sepharose (GE Healthcare) was added, and the suspension was gently mixed for 2 hours. The beads were thoroughly washed, and the attached proteins were eluted by SDS sample buffer, resolved by SDS-PAGE and analyzed by immunoblotting.

For immunoprecipitation of expressed proteins, 293T cells expressing FLAG-tagged proteins were lysed in homogenization buffer containing 1% Triton X-100 and centrifuged at 17,000 ***g*** for 10 minutes. The supernatants were immunoprecipitated with anti-FLAG M2 affinity beads (Sigma-Aldrich). After extensive washing of the beads, the bound proteins were eluted from the affinity gels by incubation with FLAG peptide and analyzed by immunoblotting.

RNA interference

The RNA duplexes used for targeting Syn5 (5′-ggaaauugaagagcuaaca-3′ and 5′-uagccucaacaaacaaauu-3′, which correspond to positions 390-408 and 432-450 relative to the start codon, respectively), Sec22b (5′-cagcauuggauucaaaggcua-3′, which corresponds to the position 491-511 relative to the start codon), CLIMP-63 (5′-gacaacaucgccaucuuca-3′, which corresponds to the position 748-766 relative to the start codon), and Bap31 (5′-ggugaaccuccagaacaau-3′, which corresponds to the position 237-255 relative to the start codon) were purchased from Japan Bioservice. Transfection was performed using Oligofectamine (Invitrogen) according to the manufacturer's protocol.

MT sedimentation assay

Polymerization of porcine brain tubulin (Cytoskeleton Inc.) was performed according to the manufacturer's instructions. Purified 2 μg of MBP or MBP fusion proteins were incubated for 30 minutes at 26℃ without or with 20 μg of polymerized tubulin in the presence of 80 μM taxol (Wako Chemical) in buffer containing 80 mM PIPES (pH 6.8), 2 mM $MgCl_2$, 0.5 mM EGTA, 0.1% Triton X-100 and 50 μM GTP. After incubation, the reaction mixtures were layered onto cushion buffer containing 80 mM PIPES (pH 6.8), 1 mM $MgCl_2$, 1 mM EGTA, 0.1% Triton X-100 and 60% glycerol with 20 μM taxol. Samples were centrifuged at 100,000 ***g*** for 40 minutes at 26℃. Equal protein amounts of the supernatant and pellet fractions were subjected to SDS-PAGE and analyzed by immunoblotting with anti-MBP antisera (New England Biolabs) and an anti-α-tubulin antibody.

Acknowledgements

We thank Mr S. Koyama for his initial contribution to the beginning of this study. We thank Dr T. Rapoport at the Harvard Medical School, Boston, MA, and Dr H. P. Hauri at the University of Basel, Switzerland, for the kind gifts of materials, and Ms K. Okada for her technical assistance. We are grateful to Dr V. Malhotra and Dr J. van Galen at the CRG in Barcelona, Spain for comments on the manuscript.

Funding

This work was supported in part by Grants-in-Aid for Scientific Research [grant numbers 22020034 and 20370050 to M.T.] from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.105304/-/DC1

References

Amessou, M., Fradagrada, A., Falguières, T., Lord, J. M., Smith, D. C., Roberts, L. M., Lamaze, C. and Johannes, L. (2007). Syntaxin 16 and syntaxin 5 are required for efficient retrograde transport of several exogenous and endogenous cargo proteins. *J. Cell Sci.* **120**, 1457-1468.

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.

Baumann, O. and Walz, B. (2001). Endoplasmic reticulum of animal cells and its organization into structural and functional domains. *Int. Rev. Cytol.* **205**, 149-214.

Bentley, M., Liang, Y., Mullen, K., Xu, D., Sztul, E. and Hay, J. C. (2006). SNARE status regulates tether recruitment and function in homotypic COPII vesicle fusion. *J. Biol. Chem.* **281**, 38825-38833.

Boström, P., Andersson, L., Rutberg, M., Perman, J., Lidberg, U., Johansson, B. R., Fernandez-Rodriguez, J., Ericson, J., Nilsson, T., Borén, J. et al. (2007). SNARE proteins mediate fusion between cytosolic lipid droplets and are implicated in insulin sensitivity. *Nat. Cell Biol.* **9**, 1286-1293.

Dabora, S. L. and Sheetz, M. P. (1988). The microtubule-dependent formation of a tubulovesicular network with characteristics of the ER from cultured cell extracts. *Cell* **54**, 27-35.

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

Du, Y., Ferro-Novick, S. and Novick, P. (2004). Dynamics and inheritance of the endoplasmic reticulum. *J. Cell Sci.* **117**, 2871-2878.

English, A. R., Zurek, N. and Voeltz, G. K. (2009). Peripheral ER structure and function. *Curr. Opin. Cell Biol.* **21**, 596-602.

Friedman, J. R., Webster, B. M., Mastronarde, D. N., Verhey, K. J. and Voeltz, G. K. (2010). ER sliding dynamics and ER-mitochondrial contacts occur on acetylated microtubules. *J. Cell Biol.* **190**, 363-375.

Gao, H., Sellin, L. K., Pütz, M., Nickel, C., Imgrund, M., Gerke, P., Nitschke, R., Walz, G. and Kramer-Zucker, A. G. (2009). A short carboxy-terminal domain of polycystin-1 reorganizes the microtubular network and the endoplasmic reticulum. *Exp. Cell Res.* **315**, 1157-1170.

Geng, L., Boehmerle, W., Maeda, Y., Okuhara, D. Y., Tian, X., Yu, Z., Choe, C. U., Anyatonwu, G. I., Ehrlich, B. E. and Somlo, S. (2008). Syntaxin 5 regulates the endoplasmic reticulum channel-release properties of polycystin-2. *Proc. Natl. Acad. Sci. USA* **105**, 15920-15925.

Grigoriev, I., Gouveia, S. M., van der Vaart, B., Demmers, J., Smyth, J. T., Honnappa, S., Splinter, D., Steinmetz, M. O., Putney, J. W., Jr, Hoogenraad, C. C. et al. (2008). STIM1 is a MT-plus-end-tracking protein involved in remodeling of the ER. *Curr. Biol.* **18**, 177-182.

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.

Hay, J. C., Chao, D. S., Kuo, C. S. and Scheller, R. H. (1997). Protein interactions regulating vesicle transport between the endoplasmic reticulum and Golgi apparatus in mammalian cells. *Cell* **89**, 149-158.

Hirose, H., Arasaki, K., Dohmae, N., Takio, K., Hatsuzawa, K., Nagahama, M., Tani, K., Yamamoto, A., Tohyama, M. and Tagaya, M. (2004). Implication of ZW10 in membrane trafficking between the endoplasmic reticulum and Golgi. *EMBO J.* **23**, 1267-1278.

Hong, W. (2005). SNAREs and traffic. *Biochim. Biophys. Acta* **1744**, 120-144.

Hu, J., Shibata, Y., Zhu, P. P., Voss, C., Rismanchi, N., Prinz, W. A., Rapoport, T. A. and Blackstone, C. (2009). A class of dynamin-like GTPases involved in the generation of the tubular ER network. *Cell* **138**, 549-561.

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.

Iinuma, T., Aoki, T., Arasaki, K., Hirose, H., Yamamoto, A., Samata, R., Hauri, H. P., Arimitsu, N., Tagaya, M. and Tani, K. (2009). Role of syntaxin 18 in the organization of endoplasmic reticulum subdomains. *J. Cell Sci.* **122**, 1680-1690.

Jahn, R. and Scheller, R. H. (2006). SNAREs–engines for membrane fusion. *Nat. Rev. Mol. Cell Biol.* **7**, 631-643.

Klopfenstein, D. R., Kappeler, F. and Hauri, H. P. (1998). A novel direct interaction of endoplasmic reticulum with microtubules. *EMBO J.* **17**, 6168-6177.

Klopfenstein, D. R., Klumperman, J., Lustig, A., Kammerer, R. A., Oorschot, V. and Hauri, H. P. (2001). Subdomain-specific localization of CLIMP-63 (p63) in the endoplasmic reticulum is mediated by its luminal α-helical segment. *J. Cell Biol.* **153**, 1287-1300.

Latterich, M., Fröhlich, K. U. and Schekman, R. (1995). Membrane fusion and the cell cycle: Cdc48p participates in the fusion of ER membranes. *Cell* **82**, 885-893.

Lavoie, C., Chevet, E., Roy, L., Tonks, N. K., Fazel, A., Posner, B. I., Paiement, J. and Bergeron, J. J. (2000). Tyrosine phosphorylation of p97 regulates transitional endoplasmic reticulum assembly in vitro. *Proc. Natl. Acad. Sci. USA* **97**, 13637-13642.

Lee, C. and Chen, L. B. (1988). Dynamic behavior of endoplasmic reticulum in living cells. *Cell* **54**, 37-46.

Lee, C., Ferguson, M. and Chen, L. B. (1989). Construction of the endoplasmic reticulum. *J. Cell Biol.* **109**, 2045-2055.

Lu, L., Ladinsky, M. S. and Kirchhausen, T. (2009). Cisternal organization of the endoplasmic reticulum during mitosis. *Mol. Biol. Cell* **20**, 3471-3480.

Lynes, E. M. and Simmen, T. (2011). Urban planning of the endoplasmic reticulum (ER): how diverse mechanisms segregate the many functions of the ER. *Biochim. Biophys. Acta* **1813**, 1893-1905.

Mizoguchi, T., Nakajima, K., Hatsuzawa, K., Nagahama, M., Hauri, H. P., Tagaya, M. and Tani, K. (2000). Determination of functional regions of p125, a novel mammalian Sec23p-interacting protein. *Biochem. Biophys. Res. Commun.* **279**, 144-149.

Müller, J. M. M., Shorter, J., Newman, R., Deinhardt, K., Sagiv, Y., Elazar, Z., Warren, G. and Shima, D. T. (2002). Sequential SNARE disassembly and GATE-16-GOS-28 complex assembly mediated by distinct NSF activities drives Golgi membrane fusion. *J. Cell Biol.* **157**, 1161-1173.

Nakajima, K., Hirose, H., Taniguchi, M., Kurashina, H., Arasaki, K., Nagahama, M., Tani, K., Yamamoto, A. and Tagaya, M. (2004). Involvement of BNIP1 in apoptosis and endoplasmic reticulum membrane fusion. *EMBO J.* **23**, 3216-3226.

Orso, G., Pendin, D., Liu, S., Tosetto, J., Moss, T. J., Faust, J. E., Micaroni, M., Egorova, A., Martinuzzi, A., McNew, J. A. et al. (2009). Homotypic fusion of ER membranes requires the dynamin-like GTPase atlastin. *Nature* **460**, 978-983.

Park, S. H., Zhu, P. P., Parker, R. L. and Blackstone, C. (2010). Hereditary spastic paraplegia proteins REEP1, spastin, and atlastin-1 coordinate microtubule interactions with the tubular ER network. *J. Clin. Invest.* **120**, 1097-1110.

Pendin, D., McNew, J. A. and Daga, A. (2011). Balancing ER dynamics: shaping, bending, severing, and mending membranes. *Curr. Opin. Cell Biol.* **23**, 435-442.

Prinz, W. A., Grzyb, L., Veenhuis, M., Kahana, J. A., Silver, P. A. and Rapoport, T. A. (2000). Mutants affecting the structure of the cortical endoplasmic reticulum in Saccharomyces cerevisiae. *J. Cell Biol.* **150**, 461-474.

Qian, F., Germino, F. J., Cai, Y., Zhang, X., Somlo, S. and Germino, G. G. (1997). PKD1 interacts with PKD2 through a probable coiled-coil domain. *Nat. Genet.* **16**, 179-183.

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.

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.

Roy, L., Bergeron, J. J., Lavoie, C., Hendriks, R., Gushue, J., Fazel, A., Pelletier, A., Morré, D. J., Subramaniam, V. N., Hong, W. et al. (2000). Role of p97 and syntaxin 5 in the assembly of transitional endoplasmic reticulum. *Mol. Biol. Cell* **11**, 2529-2542.

Shibata, Y., Hu, J., Kozlov, M. M. and Rapoport, T. A. (2009). Mechanisms shaping the membranes of cellular organelles. *Annu. Rev. Cell Dev. Biol.* **25**, 329-354.

Shibata, Y., Shemesh, T., Prinz, W. A., Palazzo, A. F., Kozlov, M. M. and Rapoport, T. A. (2010). Mechanisms determining the morphology of the peripheral ER. *Cell* **143**, 774-788.

Suga, K., Hattori, H., Saito, A. and Akagawa, K. (2005). RNA interference-mediated silencing of the syntaxin 5 gene induces Golgi fragmentation but capable of transporting vesicles. *FEBS Lett.* **579**, 4226-4234.

Suga, K., Saito, A., Tomiyama, T., Mori, H. and Akagawa, K. (2009). The Syntaxin 5 isoforms Syx5 and Syx5L have distinct effects on the processing of β-amyloid precursor protein. *J. Biochem.* **146**, 905-915.

Tagaya, M., Furuno, A. and Mizushima, S. (1996). SNAP prevents Mg^{2+}-ATP-induced release of N-ethylmaleimide-sensitive factor from the Golgi apparatus in digitonin-permeabilized PC12 cells. *J. Biol. Chem.* **271**, 466-470.

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 trans-Golgi network. *Mol. Biol. Cell* **15**, 4011-4022.

Terasaki, M. and Reese, T. S. (1994). Interactions among endoplasmic reticulum, microtubules, and retrograde movements of the cell surface. *Cell Motil. Cytoskeleton* **29**, 291-300.

Terasaki, M., Chen, L. B. and Fujiwara, K. (1986). Microtubules and the endoplasmic reticulum are highly interdependent structures. *J. Cell Biol.* **103**, 1557-1568.

Tsiokas, L., Kim, E., Arnould, T., Sukhatme, V. P. and Walz, G. (1997). Homo- and heterodimeric interactions between the gene products of PKD1 and PKD2. *Proc. Natl. Acad. Sci. USA* **94**, 6965-6970.

Uchiyama, K., Jokitalo, E., Kano, F., Murata, M., Zhang, X., Canas, B., Newman, R., Rabouille, C., Pappin, D., Freemont, P. et al. (2002). VCIP135, a novel essential factor for p97/p47-mediated membrane fusion, is required for Golgi and ER assembly in vivo. *J. Cell Biol.* **159**, 855-866.

Vedrenne, C. and Hauri, H. P. (2006). Morphogenesis of the endoplasmic reticulum: beyond active membrane expansion. *Traffic* **7**, 639-646.

Voeltz, G. K., Prinz, W. A., Shibata, Y., Rist, J. M. and Rapoport, T. A. (2006). A class of membrane proteins shaping the tubular endoplasmic reticulum. *Cell* **124**, 573-586.

Waterman-Storer, C. M. and Salmon, E. D. (1998). Endoplasmic reticulum membrane tubules are distributed by microtubules in living cells using three distinct mechanisms. *Curr. Biol.* **8**, 798-807.

West, M., Zurek, N., Hoenger, A. and Voeltz, G. K. (2011). A 3D analysis of yeast ER structure reveals how ER domains are organized by membrane curvature. *J. Cell Biol.* **193**, 333-346.

Wozniak, M. J. and Allan, V. J. (2006). Cargo selection by specific kinesin light chain 1 isoforms. *EMBO J.* **25**, 5457-5468.

Wozniak, M. J., Bola, B., Brownhill, K., Yang, Y. C., Levakova, V. and Allan, V. J. (2009). Role of kinesin-1 and cytoplasmic dynein in endoplasmic reticulum movement in VERO cells. *J. Cell Sci.* **122**, 1979-1989.

Xu, H., Brill, J. A., Hsien, J., McBride, R., Boulianne, G. L. and Trimble, W. S. (2002). Syntaxin 5 is required for cytokinesis and spermatid differentiation in *Drosophila*. *Dev. Biol.* **251**, 294-306.

Ye, X. and Sloboda, R. D. (1997). Molecular characterization of p62, a mitotic apparatus protein required for mitotic progression. *J. Biol. Chem.* **272**, 3606-3614.

Zhai, Y., Kronebusch, P. J., Simon, P. M. and Borisy, G. G. (1996). Microtubule dynamics at the G2/M transition: abrupt breakdown of cytoplasmic microtubules at nuclear envelope breakdown and implications for spindle morphogenesis. *J. Cell Biol.* **135**, 201-214.