TANGO1 recruits Sec16 to coordinately organize ER exit sites for efficient secretion

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Mammalian endoplasmic reticulum (ER) exit sites export a variety of cargo molecules including oversized cargoes such as collagens. However, the mechanisms of their assembly and organization are not fully understood. TANGO1L is characterized as a collagen receptor, but the function of TANGO1S remains to be investigated. Here, we show that direct interaction between both isoforms of TANGO1 and Sec16 is not only important for their correct localization but also critical for the organization of ER exit sites. The depletion of TANGO1 disassembles COPII components as well as membrane-bound ER-resident complexes, resulting in fewer functional ER exit sites and delayed secretion. The ectopically expressed TANGO1 C-terminal domain responsible for Sec16 binding in mitochondria is capable of recruiting Sec16 and other COPII components. Moreover, TANGO1 recruits membrane-bound macromolecular complexes consisting of cTAGE5 and Sec12 to the ER exit sites. These data suggest that mammalian ER exit sites are organized by TANGO1 acting as a scaffold, in cooperation with Sec16 for efficient secretion.

Introduction

Newly synthesized secretory proteins are transported from the ER to the Golgi apparatus in coatomer protein II (COPII)-coated vesicles (Miller and Schekman, 2013). COPII vesicles are generated at ER exit sites, the specialized ribosome-free domain of the ER (Palade, 1975; Orci et al., 1991; Bannykh et al., 1996), where the small GTPase Sar1 is activated by its guanine nucleotide exchange factor, Sec12 (Nakańo and Muramatsu, 1989; Barlowe and Schekman, 1993). Activated Sar1 then recruits the inner coat protein complex Sec23/24 with cargo molecules to form a prebudding complex (Aridor et al., 1998; Kuehn et al., 1998; Bi et al., 2002; Miller et al., 2002, 2003; Tabata et al., 2009; Pagant et al., 2015), which then binds to the outer coat protein complex Sec13/31 to finish the budding reaction (Yoshihisa et al., 1993; Matsuoka et al., 1998; Antonny et al., 2001; Bi et al., 2007). Although in vitro vesicle budding can be sufficiently reconstituted with these factors (Supek et al., 2002), another protein called Sec16 is essential for the function of the early secretory pathway in yeast, flies, and humans (Espenshade et al., 1995; Watson et al., 2006; Ivan et al., 2008; Sprangers and Rabouille, 2015).

Sec16 is a peripheral membrane protein that interacts with multiple COPII components (Gimeno et al., 1996; Shaywitz et al., 1997; Bhattacharyya and Glick, 2007; Hughes et al., 2009; Whittle and Schwartz, 2010; Montegna et al., 2012). Depletion or mutation of Sec16 results in the disruption of ER exit sites (Connerly et al., 2005; Watson et al., 2006; Bhattacharyya and Glick, 2007; Iinuma et al., 2007; Ivan et al., 2008), leading to the proposition that Sec16 acts as a scaffold protein (Sprangers and Rabouille, 2015). Additionally, studies on *Pichia pastoris* indicate that Sec16 regulates COPII assembly (Bharucha et al., 2013). It has also been reported that Sec16 negatively regulates Sar1 GTP hydrolysis by either inhibiting the recruitment of Sec31 to the prebudding complex or interacting with Sec24 (Kung et al., 2012; Yorimitsu and Sato, 2012).

It was recently reported that Sec16 integrates nutrient signaling such as extracellular stimuli and starvation (Farhan et al., 2008, 2010; Zacharogianni et al., 2011; Tillmann et al., 2015; Aguilera-Gomez et al., 2016). Furthermore, Sec16 seems to be involved in a variety of cellular functions, including T cell activation, oncogenesis, and glucose homeostasis, and to have a role in Parkinson's disease (Witte et al., 2011; Cho et al., 2014; Bruno et al., 2016; Wilhelmi et al., 2016). However, it has not yet been fully revealed how Sec16 is targeted to the ER exit sites to exert its function.

Transport and Golgi organization 1 (TANGO1) was initially identified by genome-wide screening in *Drosophila melanogaster* S2 cells for factors involved in secretion (Bard et al., 2006). Two isoforms of TANGO1, TANGO1L and TANGO1S, have been reported as mammalian orthologues. Both are localized at ER exit sites, where they form macromolecular membrane complexes with cTAGE5 and Sec12 (Maeda et al., 2016). TANGO1L interacts with collagen VII at its luminal SH3

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Abbreviations used: COPII, coatomer protein II; ELD, ER exit site localization domain; ERGIC, ER–Golgi intermediate compartment; FKBP, FK506-binding protein; FRAP, FKBP-rapamycin–associated protein; PRD, proline-rich domain; SIR, Sec16-interacting region; UCR, upstream conserved region.

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 216 No. 6 1731–1743
https://doi.org/10.1083/jcb.201703084

domain, and the cytoplasmic proline-rich domains (PRDs) of both TANGO1L and cTAGE5 interact with Sec23/24, serving as a cargo receptor for collagen VII (Saito et al., 2009, 2011; Saito and Katada, 2015). TANGO1L-knockout mice show chondrodysplasia caused by abnormal collagen secretion, confirming TANGO1L's function in vivo (Wilson et al., 2011). Moreover, TANGO1L has been reported to interact with Sedlin and sly1 (Venditti et al., 2012; Nogueira et al., 2014) and to recruit ER–Golgi intermediate compartment (ERGIC) membranes to the ER exit sites for collagen-containing megacarrier formation (Santos et al., 2015). Recently, TANGO1 was observed by superresolution microscopy to form ring-like structures around COPII coats (Raote et al., 2017).

In this study, we found that both TANGO1L and TANGO1S directly interact with Sec16 via the C-terminal PRD. The interaction is required not only for the correct localization of Sec16 and TANGO1 to the ER exit sites, but also for coordinating the localization and functioning of COPII components. Moreover, TANGO1 also organizes membrane-bound ER-resident protein complexes to the ER exit sites. The depletion of TANGO1 disassembles COPII components as well as ER-resident complexes, resulting in fewer functional ER exit sites and delayed secretion. These results suggest that TANGO1 acts as a scaffold in cooperation with Sec16 and organizes the ER exit site for efficient protein secretion.

Results

COPII components dissociate with TANGO1 depletion

Although Sec16 has been proposed to be an organizer or regulator of ER exit sites (Glick, 2014), much remains to be addressed regarding its function in mammalian cells. First, we confirmed that Sec16 extensively colocalizes with other COPII components at ER exit sites: immunofluorescence shows it as punctuated dots scattered throughout the cytoplasm (Fig. 1 A; Watson et al., 2006; Bhattacharyya and Glick, 2007; Iinuma et al., 2007). However, knockdown of both isoforms of TANGO1—either by siRNAs against sequences common to both TANGO1L and TANGO1S (panTANGO1 siRNA) or by mixture of siRNAs against specific sequences to each TANGO1L and TANGO1S (TANGO1L+S siRNAs)—redistributed Sec16 to the perinuclear regions, and the signals of scattered dots were significantly reduced (Fig. 1 A). Sec31, an outer coatomer of COP II vesicles, remained as dispersed spots through cytoplasm (Fig. 1 A). Quantitative analysis showed that the colocalization efficiency between Sec16 and Sec31 was markedly decreased in TANGO1-depleted cells (Fig. 1 B). Interestingly, Sec23 continued to colocalize with both Sec16 and Sec31 (Fig. 1 A), implying that part of Sec23 changes its localization to perinuclear regions without affecting punctate localization.

Although the cTAGE5 expression level was reduced by TANGO1 knockdown (Fig. 1 C), the dissociation between Sec16 and Sec31 was not observed (or was very limited) in cTAGE5-depleted cells (Fig. 1, A and B), indicating that TANGO1, but not cTAGE5, is responsible for the dissociation of Sec16 and Sec31. TANGO1S-FLAG expression in TANGO1-depleted cells recovered the association between Sec16 and Sec31 (Fig. 1, D and E), suggesting that TANGO1 is required for correct assembly of COPII components at ER exit sites.

Next, we tested how this disassembly affects secretion. Previous pulse-chase experiments indicated that TANGO1 is involved only in collagen secretion, without affecting general protein secretion (Saito et al., 2009). However, that study did not fully evaluate the function of TANGO1S. In the present study, we depleted TANGO1L and TANGO1S by transfecting with siRNAs twice and examining the transport of VSVG-ts045-GFP. VSVG-ts045-GFP that accumulates to the ER by incubation at 39.5°C (Fig. S1, left) is released after further incubation at 37°C. In control cells at 8 min, VSVG-ts045-GFP showed punctuated localization merged with Sec16 and Sec31 (Fig. 1 F, left). However, in cells treated with TANGO1 siRNAs, VSVG-ts045-GFP remained dispersed throughout the ER at this time point (Fig. 1 F, left). Further incubation at 37°C was required to observe spots in TANGO1-depleted cells (Fig. 1 F, right). Interestingly, the VSVG-ts045-GFP spots observed in TANGO1-depleted cells were mostly colocalized with both Sec16 and Sec31 (Fig. 1 F, right). After 30 min, the VSVG-ts045-GFP reached the Golgi in both control and TANGO1-depleted cells (Fig. S1, right), indicating that TANGO1 depletion does not block but delays VSVG trafficking. These data suggest that only sites that contain all COPII components are functional, and TANGO1 depletion reduced functional ER exit sites by dissociating Sec16 from Sec31 and delaying secretion.

TANGO1 is dispersed through the ER with Sec16 knockdown

We tested the effect of Sec16 depletion on TANGO1 localization. As shown in Fig. 2 A, when examined after methanol fixation, Sec16 depletion severely reduced the signal of TANGO1 at ER exit sites. Interestingly, Western blotting of corresponding cell lysates revealed that protein expression levels of both TANGO1L and TANGO1S were unchanged (Fig. 2 B). This phenomenon is reminiscent of what we found for Sec12 after cTAGE5 depletion (Saito et al., 2014). Sec12 is dispersed throughout the ER with cTAGE5 knockdown, so the signal seemed to be diminished in methanol-fixed cells. Interestingly, the signals of cTAGE5 and Sec12 were also diminished with Sec16 depletion (Fig. 2 A). Next, we examined the localization of TANGO1 in PFA-fixed cells. TANGO1 signals were still observed and partially colocalized with Sec12 in Sec16 siRNA–treated cells (Fig. 2 C). The signals seemed to be partially overlapped with reticular ER patterning of KDEL, implying that TANGO1 is dispersed through the ER with Sec16 depletion (Fig. 2, C and D).

TANGO1 directly interacts with Sec16

Because TANGO1 and Sec16 are mutually required for each other's correct localization, we next tested the possibility that the two proteins interact. We coexpressed FLAG-tagged Sec16 with HA-tagged TANGO1L or TANGO1S in 293T cells. Cell lysates were immunoprecipitated with anti-FLAG antibodies followed by elution with the FLAG peptide. We found that Sec16 interacts with both TANGO1S and TANGO1L (Fig. 3 A). To determine the interaction domain in Sec16, we made FLAG-tagged Sec16 deletion constructs (Figs. 3 B and S2 A) and assessed their binding with TANGO1S. As shown in Fig. 3 C, the 1,101- to 1,600-aa region of Sec16, corresponding to the ERES localization domain (ELD) and half of the central conserved domain, is capable of interacting with TANGO1S. Further deletion of Sec16 revealed that the ELD is sufficient to bind to TANGO1S (Fig. S2 B).

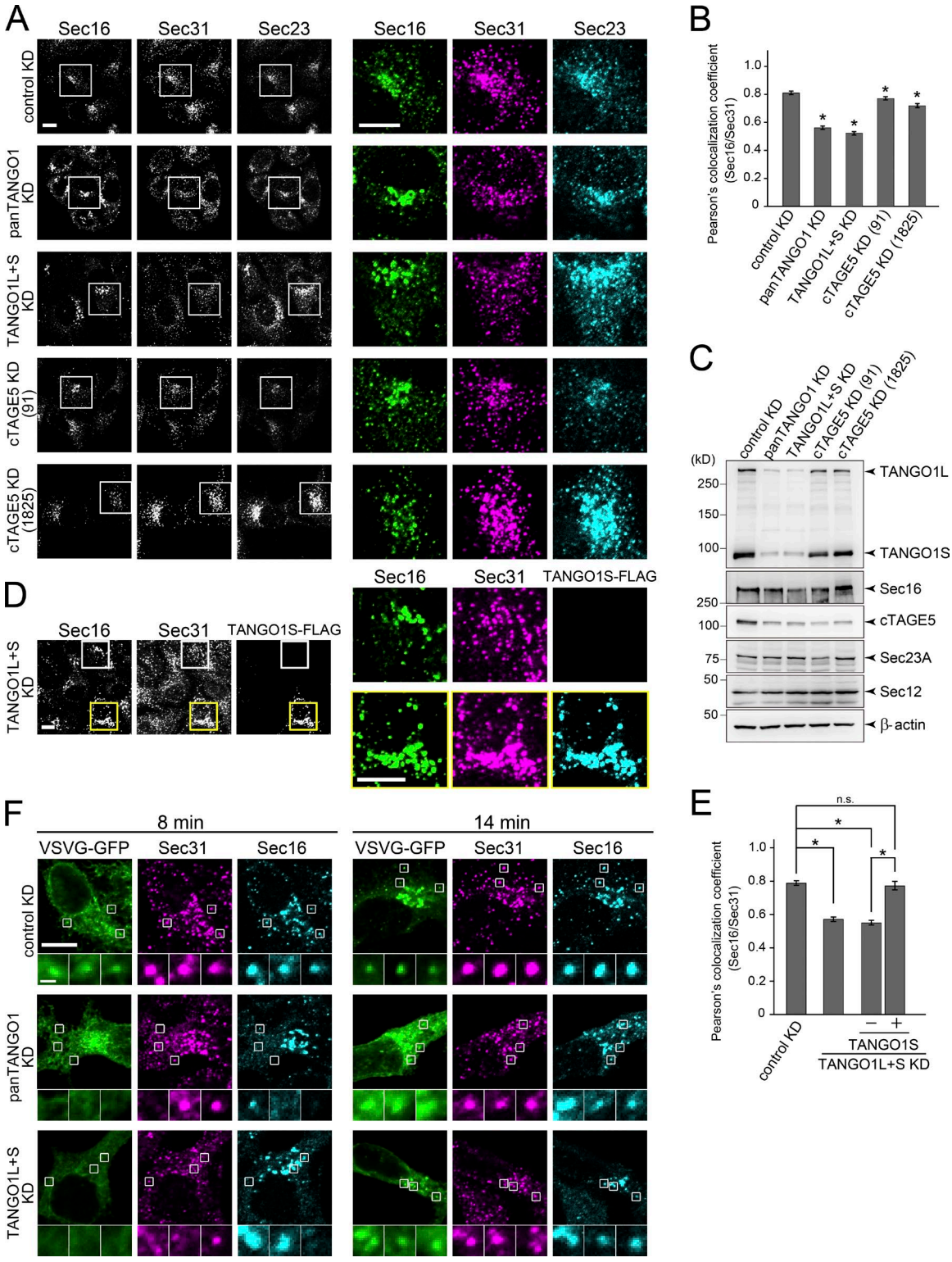

Figure 1. **TANGO1 depletion dissociates COPII components and delays secretion.** (A) HeLa cells were transfected with the indicated siRNAs. After 48 h, cells were fixed and costained with anti-Sec16-C, anti-Sec31, and anti-Sec23 (5H2) antibodies. Right, magnifications of the indicated regions on the left. Bars, 10 µm. (B) Quantification of Pearson's colocalization coefficient of A. Cells treated with control siRNA, $n = 13$; with panTANGO1 siRNA, $n = 18$; with TANGO1L and TANGO1S siRNAs, $n = 17$; with cTAGE5 (91) siRNA, $n = 16$; with cTAGE5 (1825) siRNA, $n = 18$. Error bars represent means ± SEM. *, $P < 0.05$ compared with control KD. (C) HeLa cells were transfected with the indicated siRNAs. After 48 h, proteins were extracted and subjected to SDS-PAGE, followed by Western blotting with anti-TANGO1 CC1, anti-Sec16-N, anti-cTAGE5 CC1, anti-Sec23A (11D8), anti-Sec12 (6B3), and anti β-actin antibodies. (D) HeLa cells were treated with TANGO1L and TANGO1S siRNAs. After 24 h, they were transfected with TANGO1S-FLAG construct and further cultured for 24 h. The cells were fixed and stained with anti-Sec16-C, anti-Sec31, and anti-FLAG antibodies. Right, magnifications of the indicated regions on the left (white square, TANGO1S-FLAG is not expressed; yellow square, TANGO1S-FLAG is expressed). Bars, 10 µm. (E) Quantification of Pearson's colocalization coefficient of D. For cells treated with control siRNA, $n = 17$; with TANGO1L and TANGO1S siRNAs, $n = 17$; with TANGO1L and

Next, we investigated which region of TANGO1 was responsible for the interaction with Sec16. We created recombinant proteins of TANGO1 deletions fused with GST and assessed their interaction with the FLAG-tagged 1,101- to 1,600-aa region of Sec16 purified from 293T cells (Fig. S3). We found that the PRD of TANGO1 directly interacts with the 1,101- to 1,600-aa region of Sec16 (Fig. 3 D). We made further deletions of the PRD and examined the interaction with Sec16 (Fig. S3). When the TANGO1 PRD was truncated by 60 aa at its C-terminal end, it was able to bind to Sec16 (Fig. 3 E, lane 4). However, when 120 aa was deleted from its C terminus, it failed to interact with Sec16 (Fig. 3 E, lane 5), indicating that the interaction domain of TANGO1's PRD for Sec16 lies 60–120 aa from the C terminus. We named this region (1,788–1,847 aa) of TANGO1 the Sec16-interacting region (SIR). Because TANGO1's PRD has also been reported to interact with Sec23 (Saito et al., 2009; Ma and Goldberg, 2016), we next tested the interaction with Sec23 (Fig. S3). As shown in Fig. 3 F, Sec23A is also capable of interacting with 60-aa truncated PRD (Fig. 3 F, lane 4), but the binding was severely reduced with a 120-aa deletion of PRD (Fig. 3 F, lane 5), suggesting that the major interacting domain of Sec23 corresponds to TANGO1's SIR.

Interaction between Sec16 and TANGO1 is required for their correct localization

To clarify the functional significance of the interaction between TANGO1 and Sec16, we next examined the cells with TANGO1 deletion constructs. The cells depleted of both TANGO1L and TANGO1S were transfected with various constructs of TANGO1, and their localization and effects on other COPII components were investigated. As shown in Fig. 4 A, expressed TANGO1SΔ45 and TANGO1SΔ60 were localized at punctate dots characteristic of ER exit sites and extensively merged with Sec16 and Sec31 as TANGO1S full-length constructs. Quantitative analysis showed that the colocalization efficiency between Sec16 and Sec31 was recovered on the cells with these constructs (Fig. 4 B). Conversely, the cells with TANGO1SΔ120, which cannot interact with Sec16 and Sec23 (Fig. 3, E and F), showed ER reticular patterning, and this construct failed to correct the dissociation of Sec16 and Sec31 (Fig. 4, A and B). These results strongly suggest that the domain of TANGO1 responsible for the interaction with Sec16 and Sec23 (SIR) is required for correct localization of TANGO1, Sec16, and Sec31 at the ER exit sites.

Although in vitro binding assays revealed that TANGO1 interacts directly with Sec16 (Fig. 3, D and E), the possibility that TANGO1 has a common binding region for Sec16 and Sec23 (Fig. 3, E and F) prompted us to investigate whether Sec23 is required for the correct localization of TANGO1 and Sec16 at ER exit sites. Unfortunately, it is difficult to costain endogenous TANGO1 and Sec16, because antibodies against both proteins were made in rabbits. Thus, we made use of doxycycline-inducible TANGO1S-GFP stable HeLa cell lines. We depleted Sec23A and Sec23B by siRNAs and verified knockdown efficiency by anti-Sec23 (5H2) antibody, which can recognize both Sec23A and Sec23B, as shown in Fig. S4. When both Sec23A and Sec23B are efficiently depleted, induced TANGO1S-GFP extensively colocalizes with Sec16, indicating that Sec23 is not necessary for correct localization of TANGO1 and Sec16 (Fig. 4 C). We also confirmed by immunofluorescence that endogenous TANGO1S and TANGO1L did not change their localization when Sec23 was depleted (Fig. S5). Collectively, these results suggest that interaction between TANGO1 and Sec16 is not only required for their correct localization, but is also important for functional ER exit sites.

TANGO1 scaffolds ER-resident protein complexes to the ER exit sites

We have previously reported that TANGO1 forms macromolecular ER-resident protein complexes with cTAGE5 and Sec12 at ER exit sites (Maeda et al., 2016). Because TANGO1 seems to coordinate the localization of cytosolic COPII components to the ER exit sites, we next investigated whether TANGO1 is also important for recruiting ER-resident complexes to the ER exit sites. Our previous study suggested that Sec12 localizes at ER exit sites via direct interaction with cTAGE5 (Saito et al., 2014). Thus, we examined whether TANGO1 recruits cTAGE5 to the ER exit sites or vice versa.

We depleted all of TANGO1L, TANGO1S, and cTAGE5 from the cells and checked the localization of expressed constructs. Because siRNA for TANGO1S is targeted to the 5′ UTR, the TANGO1S original construct can be used as a rescue construct, in contrast to cTAGE5, which requires an siRNA-resistant mutant. When TANGO1S-FLAG is expressed in the cells depleted of TANGO1 and cTAGE5, Sec16 recovers its association with Sec31, and TANGO1S-FLAG colocalizes with these COPII components (Fig. 5 A), suggesting that TANGO1 is capable of recruiting itself to the ER exit sites independently of cTAGE5, as long as Sec16 is present. Although expressed cTAGE5-FLAG colocalizes with Sec16 and Sec31 in cTAGE5-depleted cells (Fig. 5 A), when expressed in cells depleted of both cTAGE5 and TANGO1, it no longer colocalizes with Sec16 and Sec31 (Fig. 5 A). Moreover, the dissociation of Sec16 and Sec31 is not recovered with cTAGE5 expression. These results strongly suggest that TANGO1 recruits cTAGE5 to the ER exit sites.

Next, we examined Sec12 localization under the same conditions. We used a cTAGE5-FLAG siRNA-resistant construct lacking the epitope for the cTAGE5-CT antibody, so that expressed cTAGE5 was not recognizable by the antibody. Although Sec12 is dispersed throughout the ER in cTAGE5-depleted cells, cTAGE5-FLAG expression rescues the Sec12 signals at ER exit sites (Fig. 5 B; Saito et al., 2014). We showed previously that when TANGO1 is depleted, cTAGE5 expression was reduced and Sec12 no longer localized at ER exit sites (Maeda et al., 2016). TANGO1S expression in these cells rescues the cTAGE5 expression, leading to Sec12 relocation to the ER exit sites (Fig. 5 B). When TANGO1S is expressed in cTAGE5- and TANGO1-depleted cells, Sec12 remains dispersed throughout the ER because cTAGE5 expression is not recovered (Fig. 5 B). Interestingly, when cTAGE5 is expressed in a cTAGE5- and TANGO1-depleted environment, Sec12

TANGO1S siRNAs, in which TANGO1S-FLAG is not expressed, $n = 20$; with TANGO1L and TANGO1S siRNAs, in which TANGO1S-FLAG is expressed, $n = 12$ (analysis of variance). *, $P < 0.05$; n.s., not significant. Error bars represent means ± SEM. (F) HeLa cells twice transfected with the indicated siRNAs were transfected with VSVG-ts045-GFP. The cells were cultured at 39.5°C to accumulate the protein in the ER, then incubated for the indicated times at 37°C before fixation. Fixed cells were stained with anti-Sec16-C and anti-Sec31 antibodies. Bars: (main) 10 µm; (insets) 1 µm.

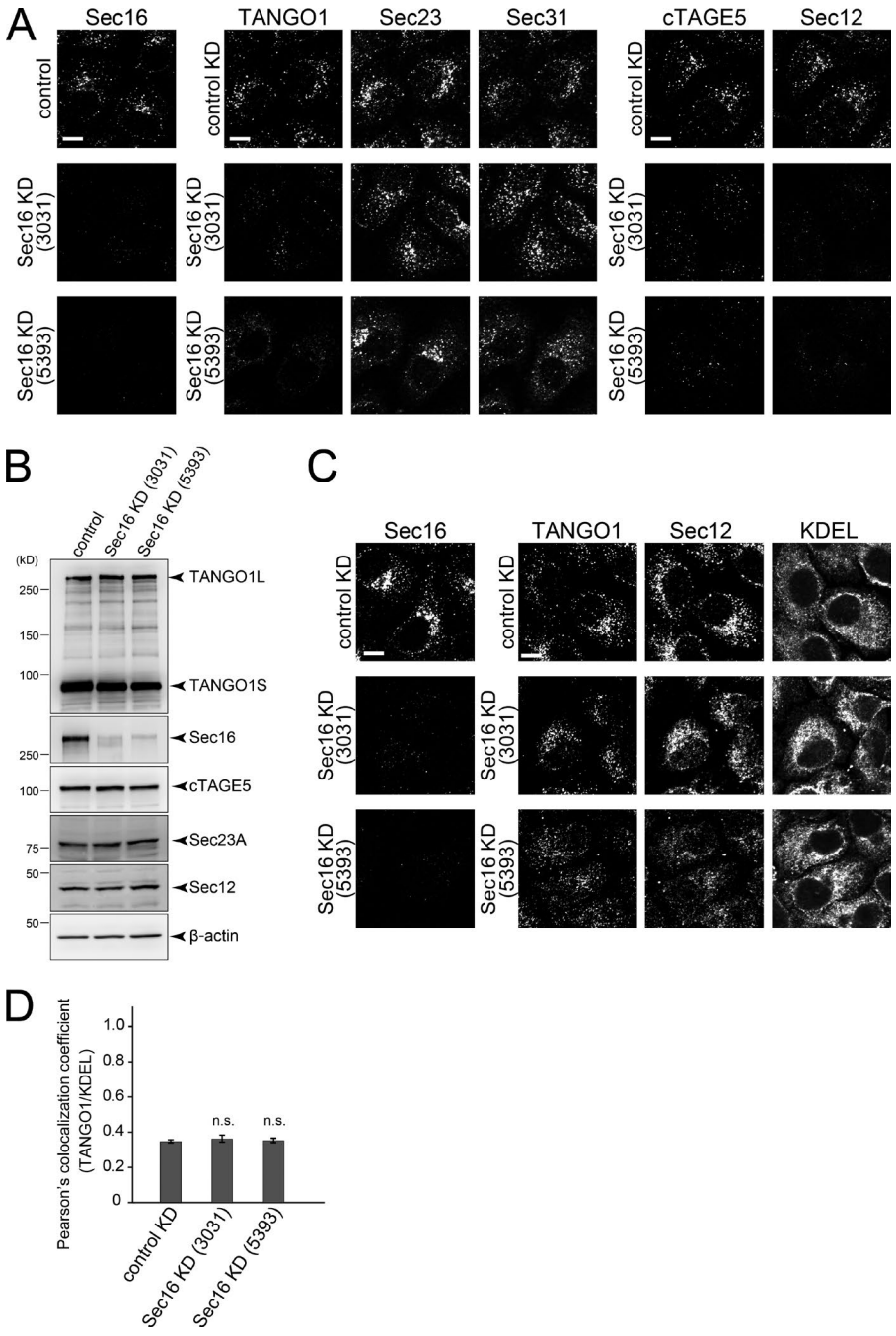

Figure 2. **TANGO1 is dispersed through the ER after Sec16 knockdown.** (A) HeLa cells were transfected with the indicated siRNA. After 72 h, cells were fixed with cold methanol and costained with anti-Sec16-C, anti-TANGO1 CT, anti-Sec23 (5H2), anti-Sec31, anti-cTAGE5 CT, and anti-Sec12 (6B3) antibodies. Bars, 10 µm. (B) HeLa cells were transfected with the indicated siRNAs. After 72 h, proteins were extracted and subjected to SDS-PAGE, followed by Western blotting with anti-TANGO1 CC1, anti-Sec16-N, anti-cTAGE5 CC1, anti-Sec23A (11D8), anti-Sec12 (6B3), and anti β-actin antibodies. (C) HeLa cells were transfected with the indicated siRNAs. After 72 h, cells were fixed with PFA and costained with anti-Sec16-C, anti-TANGO1 CT, anti-Sec12 (6B3), and anti-KDEL antibodies. Bars, 10 µm. (D) Quantification of Pearson's colocalization coefficient of C. For cells treated with control siRNA, $n = 13$; with Sec16 (3031) siRNA, $n = 12$; with Sec16 (5393) siRNAs, $n = 10$. Error bars represent means ± SEM. n.s., not significant compared with control KD.

signals fail to accumulate where cTAGE5 locates, confirming that cTAGE5 fails to recover functional ER exit sites, with dissociated localization of Sec16, Sec31, cTAGE5, and Sec12. These results strongly suggest that TANGO1 functions as an organizer for membrane-bound protein complexes and recruits them to ER exit sites.

TANGO1 is capable of recruiting Sec16 and other COPII components

Although Sec16 is considered to be an organizer of the ER exit sites, the fact that Sec16 is a peripheral membrane protein suggests that it must be first recruited to the ER exit site to exert its function of scaffolding other COPII components. This is in contrast to both TANGO1L and TANGO1S, which are membrane-spanning proteins localized at ER exit sites. Because our results showed that TANGO1 organizes membrane-bound proteins at ER exit sites, we decided to investigate whether TANGO1 was also capable of recruiting Sec16. To this end, we conducted a rapamycin-dependent distribution assay. This assay depends on the properties of two proteins, FK506-binding protein (FKBP) and FKBP-rapamycin–associated protein (FRAP), also known as mTOR, which interact only in the presence of rapamycin. We expressed a Tomm20-GFP-FRAP construct for mitochondria targeting. In addition, we coexpressed TANGO1 deletion constructs fused with an HA tag and FKBP (Fig. 6 A). Without rapamycin, even though Tomm20-GFP-FRAP was efficiently targeted to mitochondria, both TANGO1 PRD-HA-FKBP and TANGO1 PRDΔ120-HA-FKBP were localized in the cytoplasm and the nucleus (Fig. 6 B). After 2-h treatment with rapamycin, both proteins were extensively

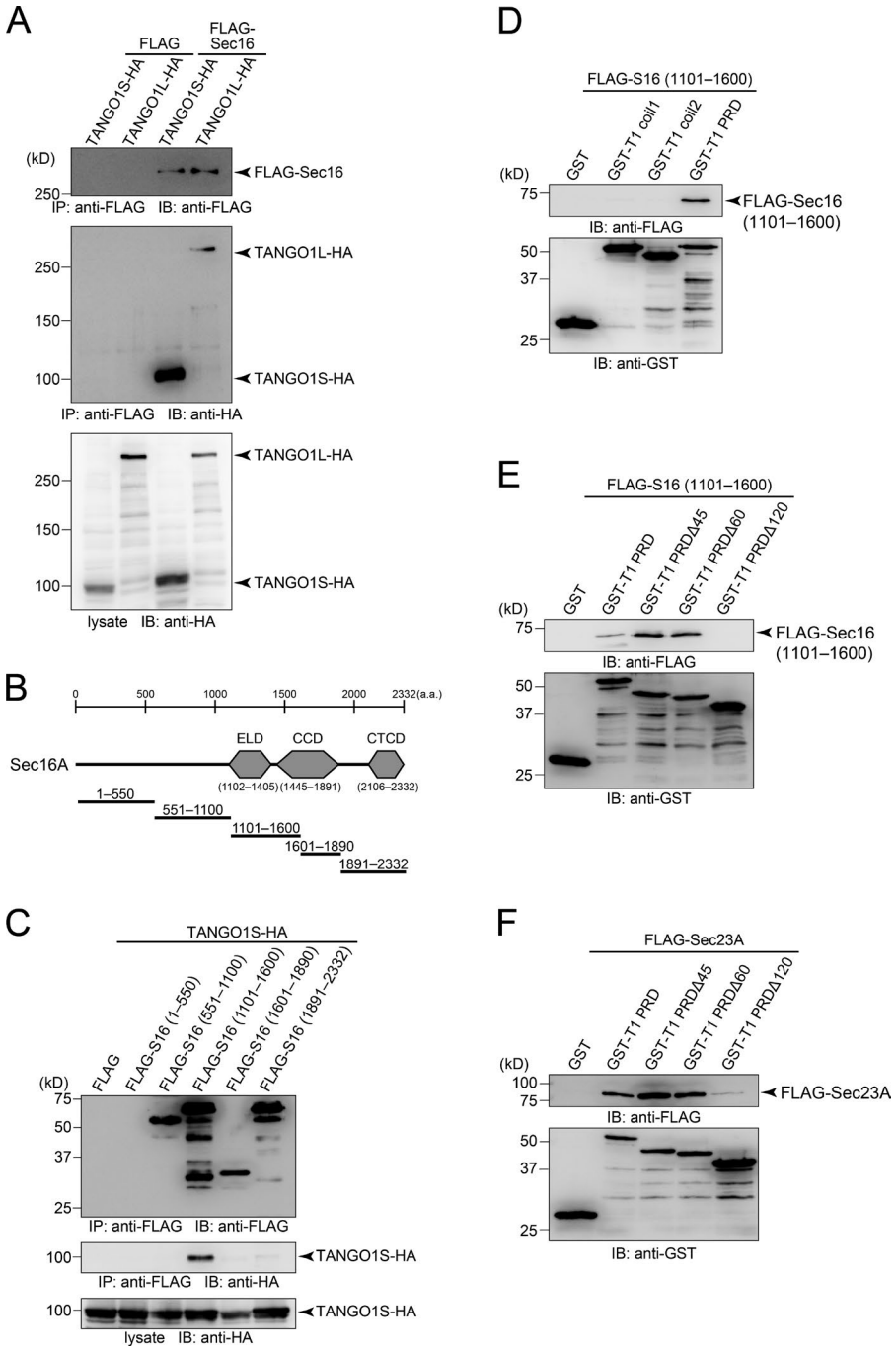

Figure 3. **TANGO1 interacts with Sec16.** (A) 293T cells were transfected with TANGO1S-HA or TANGO1L-HA with FLAG-Sec16 constructs as indicated. Cell lysates were immunoprecipitated with anti-FLAG antibody and eluted with a FLAG peptide. Eluates were then subjected to SDS-PAGE followed by Western blotting with anti-FLAG and anti-HA antibodies. (B) Schematic representation of human Sec16A domain organization. CCD, central conserved domain; CTCD, C-terminal conserved domain. (C) 293T cells were transfected with FLAG-Sec16 (1–550 aa), FLAG-Sec16 (551–1,100 aa), FLAG-Sec16 (1,101–1,600 aa), FLAG-Sec16 (1,601–1,890 aa), or FLAG-Sec16 (1,891–2,332 aa) with TANGO1S-HA constructs as indicated. Cell lysates were immunoprecipitated with anti-FLAG antibody and eluted with a FLAG peptide. Eluates were then subjected to SDS-PAGE followed by Western blotting with anti-FLAG and anti-HA antibodies. (D) Recombinant GST, GST-tagged TANGO1-coil1 (1,211–1,440 aa), GST-tagged TANGO1-coil2 (1,441–1,650 aa), or GST-tagged TANGO1-PRD (1,651–1,907 aa) were immobilized to glutathione Sepharose resin and incubated with FLAG-Sec16 (1,101–1,600 aa). Resins were washed and eluted with glutathione. Eluted proteins were subjected to SDS-PAGE followed by Western blotting with anti-FLAG and anti-GST antibodies. (E and F) GST, GST-tagged TANGO1-PRD (1,651–1,907 aa), GST-tagged TANGO1-PRDΔ45 (1,651–1,862 aa), GST-tagged TANGO1-PRDΔ60 (1,651–1,847 aa), or GST-tagged TANGO1-PRDΔ120 (1,651–1,787 aa) were immobilized to glutathione Sepharose resin and incubated with FLAG-Sec16 (1,101–1,600 aa; E) or FLAG-Sec23A (F). Resins were washed and eluted with glutathione. Eluted proteins were subject to SDS-PAGE followed by Western blotting with anti-FLAG and anti-GST antibodies.

colocalized with Tomm20-GFP-FRAP constructs on the mitochondria. We then checked the localization of Sec16. As shown in Fig. 6 B, Sec16 was extensively recruited to mitochondria when TANGO1 PRD-HA-FKBP was expressed in cells in the presence of rapamycin. Conversely, in the cells with TANGO1 PRDΔ120-HA-FKBP, which lacks the Sec16 interaction domain (Fig. 3 E), Sec16 remained localized to the punctuated dots characteristic of ER exit sites (Fig. 6 B). Moreover, another COPII component, Sec31, seemed to be recruited to the mitochondrial membrane, though with less efficiency than Sec16, when TANGO1 PRD-HA-FKBP was expressed in the presence of rapamycin (Fig. 6 C). These results suggest that TANGO1 is capable of recruiting Sec16 and other cytoplasmic COPII components to the ER exit sites. Collectively, this is evidence that TANGO1 recruits both membrane-bound and peripheral proteins and scaffolds them for correct functioning at ER exit sites.

Discussion

TANGO1L has been extensively characterized as a collagen cargo receptor at ER exit sites (Malhotra and Erlmann, 2015; Saito and Katada, 2015). The luminal SH3 domain of TANGO1L interacts with collagen VII or HSP47 associated with collagens and facilitates collagen export from the ER (Saito et al., 2009; Ishikawa et al., 2016). In this study, we found that TANGO1, consisting of TANGO1L and TANGO1S, is partly involved in general protein secretion.

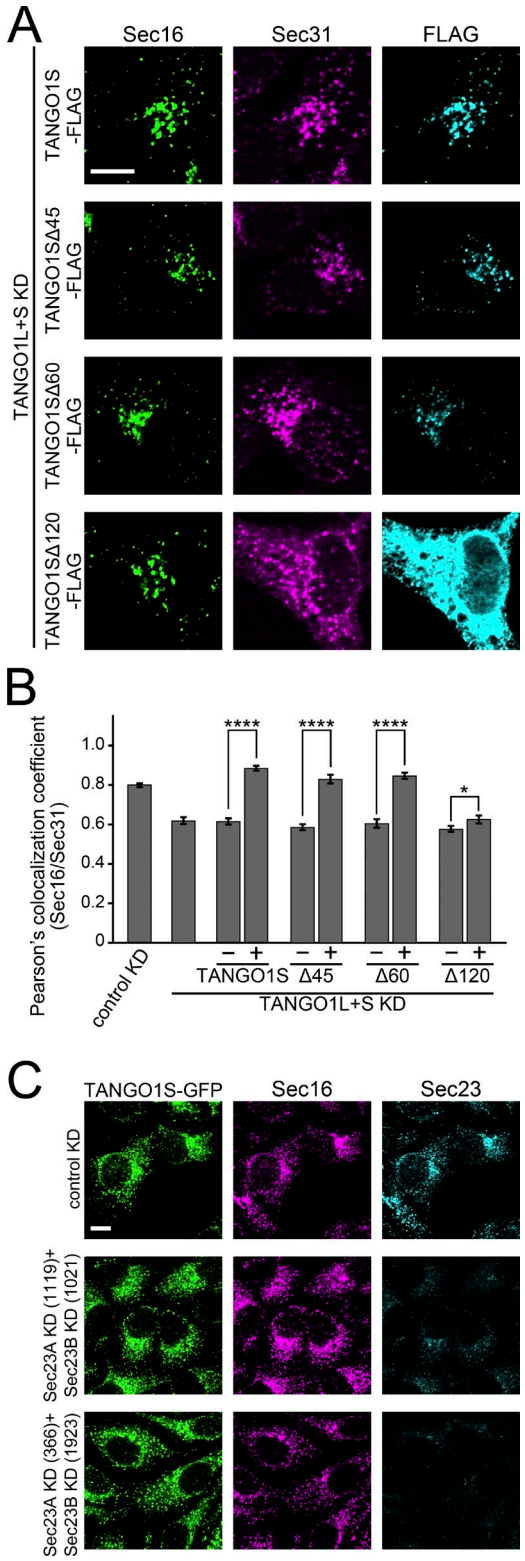

Figure 4. **Interaction between TANGO1 and Sec16 is required for correct localization of both proteins and other COPII components.** (A) HeLa cells were treated with TANGO1L and TANGO1S siRNAs. After 24 h, TANGO1S-FLAG, TANGO1SΔ45-FLAG, TANGO1SΔ60-FLAG, or TANGO1SΔ120-FLAG constructs were transfected and further cultured for 24 h. The cells were fixed and stained with anti-Sec16-C, anti-Sec31, and anti-FLAG antibodies. Bars, 10 µm. (B) Quantification of Pearson's colocalization coefficient of A. For cells treated with control siRNA, n = 18; with TANGO1L and TANGO1S siRNAs, n = 16; with TANGO1L and TANGO1S siRNAs, in which TANGO1S-FLAG is not expressed, n = 18; with TANGO1L and TANGO1S siRNAs, in which TANGO1S-FLAG is expressed, n = 10; with TANGO1L and TANGO1S siRNAs, in which TANGO1SΔ45-FLAG is not expressed, n = 25; with TANGO1L and TANGO1S siRNAs, in which TANGO1SΔ45-FLAG is expressed, n = 10; with TANGO1L and TANGO1S siRNAs, in which TANGO1SΔ60-FLAG is not expressed, n = 19; with TANGO1L and TANGO1S siRNAs in which TANGO1SΔ60-FLAG is expressed, n = 10; with TANGO1L and TANGO1S siRNAs, in which TANGO1SΔ120-FLAG is not expressed, n = 27; with TANGO1L and TANGO1S siRNAs, in which TANGO1SΔ120-FLAG is expressed, n = 10 (analysis of variance). *, $P < 0.05$; ****, $P < 0.0001$. Error bars represent means ± SEM. (C) Doxycycline-inducible stable HeLa cell line for TANGO1S-GFP was treated with Sec23A and Sec23B siRNAs. After 24 h, TANGO1S-GFP was induced and cells were further cultured for 24 h. The cells were fixed and stained with anti–Sec16-C and anti-Sec23 (5H2) antibodies. Bars, 10 µm.

We showed that VSVG trafficking is delayed by the depletion of both TANGO1L and TANGO1S. Moreover, we found that TANGO1 depletion compromises COPII protein integrity, observed as reduced colocalization efficiency between Sec16 and Sec31. Cargoes seem to be exported by functional ER exit sites characterized by the presence of each COPII component, including Sec16 and Sec31. TANGO1 depletion decreased the number of functional ER exit sites, leading to delayed secretion. These results indicate that TANGO1 is involved in the coordination of COPII components at ER exit sites for efficient secretion.

In this study, we showed that TANGO1 directly interacts with Sec16 and mapped the interaction domains of both proteins to regions (1,788–1,847 aa) in PRD of TANGO1 (SIR) and ELD of Sec16. In addition, SIR in TANGO1 seems to be also important for Sec23 binding. TANGO1's PRD has been shown by yeast two-hybrid system to interact with Sec23 (Saito et al., 2009), and recent structural analysis suggested that multiple Sec23 molecules can bind to TANGO1's repeated PPP motifs in PRD (Ma and Goldberg, 2016). Consistent with that article, our in vitro binding assay showed that TANGO1 PRDΔ60 (1,651–1,847 aa), which contains five of seven PPP motifs, efficiently interacts with Sec23, although PRDΔ120 (1,651–1,787 aa), which has only two PPP motifs, barely binds to Sec23 (Fig. 3 F).

SIR in TANGO1 is important for TANGO1's localization to the ER exit sites. However, it is not likely that Sec23 is involved in the regulation of TANGO1's localization, because Sec23 depletion has no effect on the localization of either Sec16 or TANGO1. These results strongly suggest that Sec16 binding is required for correct localization of TANGO1 to the ER exit sites.

Sec16 has been proposed to be an organizer or regulator, as it binds and accumulates multiple COPII components to the ER exit sites (Connerly et al., 2005; Bharucha et al., 2013; Sprangers and Rabouille, 2015); however, the fact that Sec16 is a peripheral membrane protein indicates that there must be some mechanism to recruit the protein to the membranes. ELD was previously shown to be required for ER exit site localization of Sec16 in *Drosophila* and humans (Ivan et al., 2008; Hughes et al., 2009), but the mechanism was not revealed. The current study reveals that TANGO1 interacts with Sec16 via ELD. The fact that TANGO1, regardless of isoform, is an integral membrane protein strongly suggests that membrane association of Sec16 is mediated at least in part by TANGO1. Surprisingly, TANGO1 depletion did not cause Sec16 dispersion to

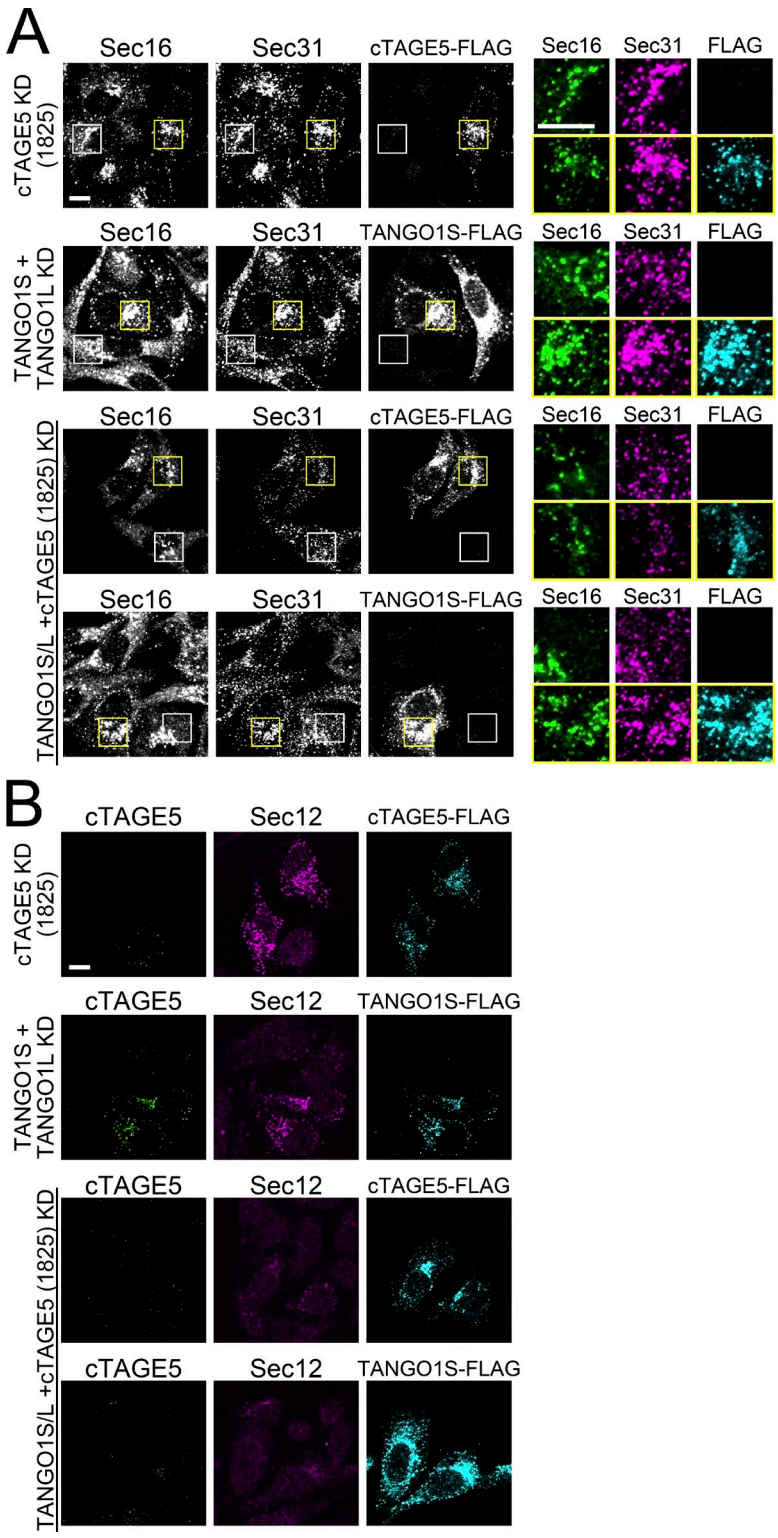

Figure 5. **TANGO1 organizes membrane-bound ER exit site components.** (A) HeLa cells were transfected with the indicated siRNAs. After 24 h, TANGO1S-FLAG or cTAGE5-FLAG constructs were transfected and further cultured for 24 h. The cells were fixed and stained with anti-Sec16-C, anti-Sec31, and anti-FLAG antibodies. Right, magnifications of the indicated regions on the left (white square, TANGO1S-FLAG or cTAGE5-FLAG are not expressed; yellow square, TANGO1S-FLAG or cTAGE5-FLAG are expressed). Bars, 10 µm. (B) HeLa cells were transfected with the indicated siRNAs. After 24 h, TANGO1S-FLAG or cTAGE5-FLAG constructs were transfected and further cultured for 24 h. The cells were fixed and stained with anti-cTAGE5 CT, anti-Sec12 (6B3), and anti-FLAG antibodies. Bars, 10 µm.

the cytosol: it still localized to the perinuclear regions, implying that Sec16 still associates with membranes. One explanation for this is that Sec16's ELD might recognize factors other than TANGO1, such as certain lipids at the ER exit site membrane. If so, TANGO1 depletion might lead to Sec16 relocation to the domains enriched in those factors. In any case, we showed that ectopically expressed TANGO1 alone efficiently recruits Sec16. Thus, we conclude that interaction with TANGO1 recruits Sec16 to the ER exit sites.

As discussed, TANGO1 and Sec16 are mutually required for their correct localization to the ER exit sites. Sec16 has been shown to form homo-oligomers (Bhattacharyya and Glick, 2007; Yorimitsu and Sato, 2012). It is interesting to speculate that the oligomerization property of Sec16 might be involved in the concentration of TANGO1 to the ER exit sites. Further work is needed to explore this possibility. In this context, it is noteworthy that TFG-1, an interactor of Sec16, forms homo-octamers and is involved in the association of

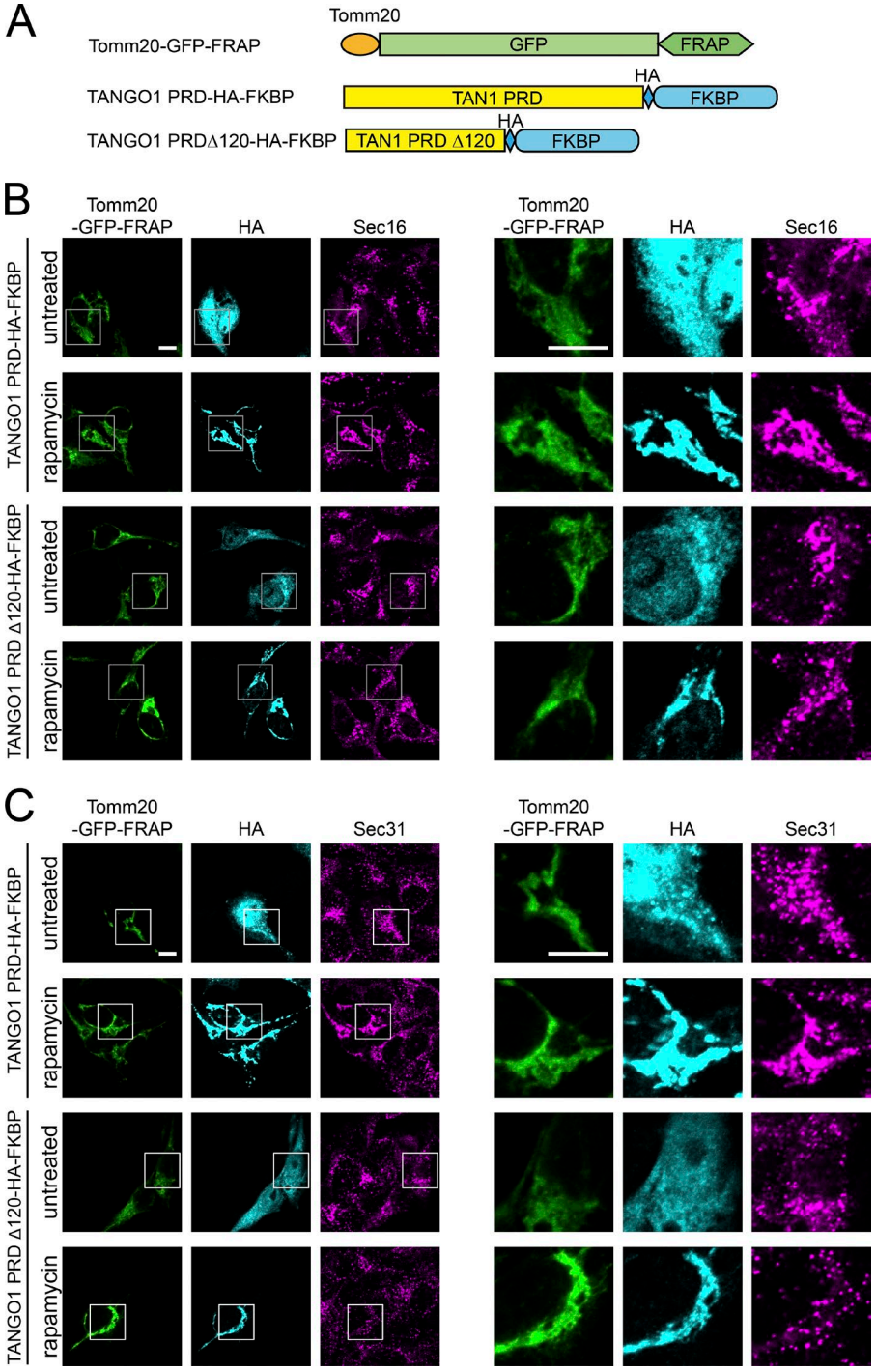

Figure 6. **TANGO1 proline-rich domain recruits Sec16 and other COPII components.** (A) Schematic representation of the constructs used in this study. Tomm20 (1–33 aa)-GFP-FRAP, TANGO1 PRD domain (1,651–1,907 aa)-HA-FKBP, TANGO1 PRD Δ120 (1,651–1,787 aa)-HA-FKBP. (B and C) HeLa cells were treated with TANGO1L and TANGO1S siRNAs. After 24 h, the Tomm20-GFP-FRAP construct and TANGO1 PRD-HA-FKBP or TANGO1 PRD Δ120-HA-FKBP constructs were transfected, and cells were further cultured for 24 h. The cells were incubated for another 2 h with or without 200 nM rapamycin. After fixation, the cells were stained with anti-HA and anti–Sec16-C (B) or anti-Sec31 (C) antibodies. Right, magnifications of the indicated regions on the left. Bars, 10 µm.

small functional ER exit sites into large structures required for collagen export (Witte et al., 2011; Johnson et al., 2015; McCaughey et al., 2016). In any case, our current analysis suggests that TANGO1, in cooperation with Sec16, is required for maintaining functional ER exit sites characterized by the presence of each COPII component.

We have previously shown that TANGO1 forms two different membrane-bound macromolecular complexes at ER exit sites (Maeda et al., 2016). Either TANGO1L or TANGO1S interacts with cTAGE5 oligomers, the cTAGE5 oligomers then bind to the corresponding amount of Sec12, forming 900- and 700-kD complexes. We have also reported that Sec12 localization to the ER exit sites is dependent on its direct interaction with cTAGE5 (Saito et al., 2014). Here, we found that cTAGE5 is recruited to the ER exit sites by direct interaction with TANGO1. Thus, TANGO1 is required for cTAGE5 and Sec12 to localize at the ER exit sites.

Based on these results, we propose a model that TANGO1, in cooperation with Sec16, acts as scaffold and organizes the ER exit site for efficient secretion (Fig. 7 A). Supporting results for this model are as follows: (a) TANGO1 and Sec16 are mutually required for their correct localization to the ER exit sites; (b) TANGO1 is important for recruiting components of the membrane-bound ER-resident complex (i.e., cTAGE5

and Sec12) to the ER exit sites; (c) TANGO1 and Sec16 are independently necessary for the coordination of the COPII components at the ER exit sites; and (d) disruption of the coordination by either TANGO1 or Sec16 depletion causes at least delayed secretion from the ER.

Cytoplasmic coiled-coil domains of both TANGO1L and TANGO1S shares sequence called TEER, which is important for recruiting ERGIC membranes to the ER exit sites (Santos et al., 2015). Further investigation is required to uncover the relationship between two distinct functions of cytoplasmic domains of TANGO1, to recruit Sec16 via SIR and to recruit ERGIC membranes via TEER.

It is also interesting to evaluate current findings from an evolutionary perspective. The core components of COPII-vesicle formation including Sar1, Sec12, Sec23/24, Sec13/31, and Sec16 are all conserved from budding yeast. In *Saccharomyces cerevisiae,* Sec12 is dispersed through the ER, in contrast to the concentrated localization of Sec16, Sec23/24, and Sec13/31 at the ER exit sites (Okamoto et al., 2012). Interestingly, modest concentrations of Sar1 around ER exit sites that are devoid of COPII proteins have been observed by superresolution confocal live imaging microscopy (Kurokawa et al., 2016). These results imply that in spite of the dispersed localization of activator Sec12, Sar1 has to be concentrated around ER exit sites to function (Fig. 7 B).

In *P. pastoris*, a budding yeast, Sec12 is concentrated at the ER exit sites by its direct interaction with Sec16 (Montegna et al., 2012). Sar1 is also concentrated at the ER exit sites in this species (Fig. 7 C; Soderholm et al., 2004). These two types of yeast share the upstream conserved region (UCR) required for ER exit site localization of Sec16, and it has been reported for *P. pastoris* that UCR interacts with COPII components and that this interaction is required for Sec16 localization to the ER exit sites (Bharucha et al., 2013). Interestingly, in spite of functional resemblance between UCR in yeast and ELD in humans, sequence similarity is not observed, implying that the mechanisms of Sec16 association to the membrane are different among these species. Further, TANGO1 is only conserved in metazoans (Saito et al., 2009). TANGO1 might have evolved to recruit Sec16 to ER exit sites by the direct interaction between SIR and ELD. Moreover, human TANGO1 interacts with cTAGE5 and recruits multiple Sec12 molecules in association with cTAGE5, compared with the Sec12 recruitment by direct interaction with Sec16 observed in *P. pastoris* (Fig. 7 D). Thus, it would be interesting to speculate that higher eukaryotes introduced ER-resident complexes consisting of TANGO1 and cTAGE5 for recruiting multiple Sec12 molecules for the efficient production of Sar1 in the vicinity of ER exit sites organized by Sec16. It is noteworthy that *Drosophila* only has the long isoform of TANGO1 and it seems not have cTAGE5 counterparts. It might be worth testing whether fly TANGO1 interacts with Sec12 and Sec16, and if it is able to oligomerize.

During the preparation of this manuscript, it was reported that *Drosophila* TANGO1 also functions as an organizer; however, the mechanism by which TANGO1 organizes ER exit sites remained unclear (Liu et al., 2017). Our present study provides the evidence that TANGO1 directly interacts with Sec16 and, in cooperation with Sec16, acts as a scaffold for efficient protein secretion.

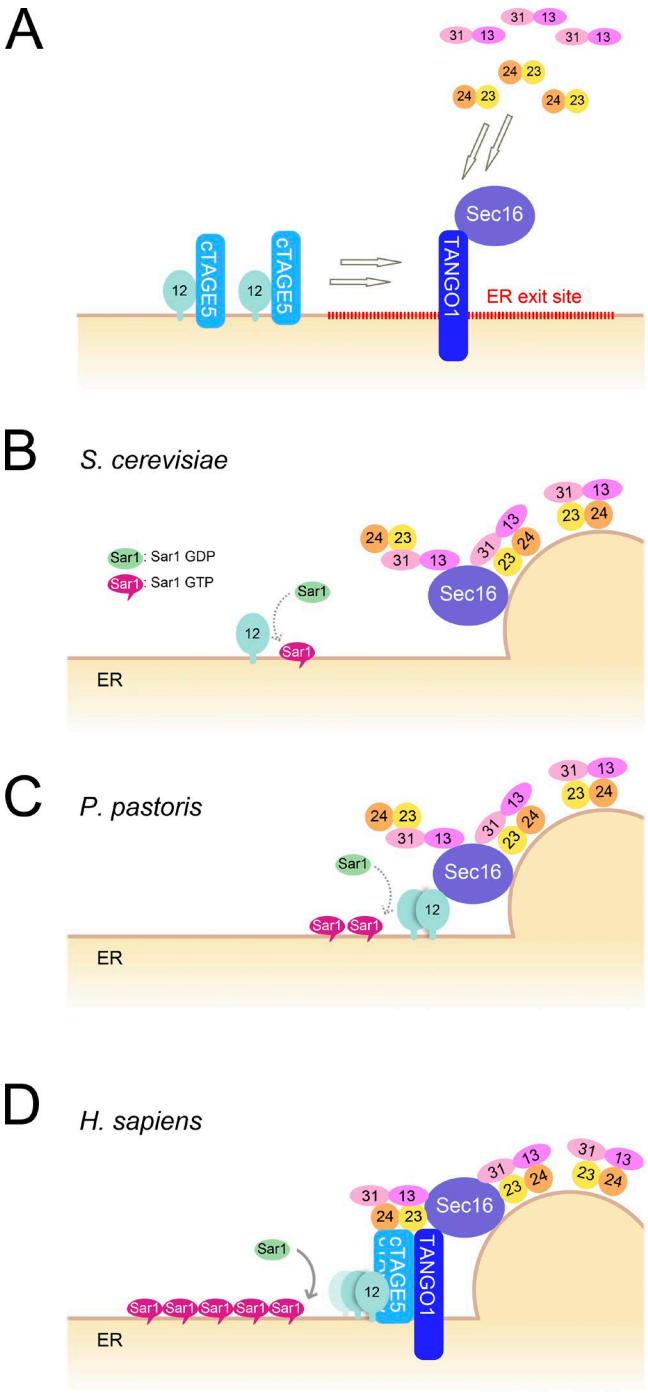

Figure 7. **A model for mammalian ER exit site organization by TANGO1 and Sec16.** (A) TANGO1 and Sec16 are mutually required for their localization to the ER exit sites. TANGO1 recruits membrane-bound ER-resident proteins to form macromolecular complexes. TANGO1 and Sec16 then interact with COPII components and localize them to the ER exit sites. (B) A model depicting ER exit sites of *S. cerevisiae*. Sec16 is recruited to the ER exit sites via the UCR. Sec12 is dispersed throughout the ER. Sar1 shows some accumulation at the rim of COPII-coated vesicles. (C) A model depicting ER exit sites of *P. pastoris*. Sec16 is recruited to the ER exit sites via interaction with COPII proteins. Sec12 is concentrated to the ER exit sites via interaction with Sec16. Sar1 is accumulated to the ER exit sites. (D) A model depicting ER exit sites of *Homo sapiens*. TANGO1 and Sec16 scaffold COPII components and ER-resident complexes at the ER exit sites. Sec16 is recruited to the ER exit sites via ELD by direct interaction with SIR of TANGO1. Sar1 is efficiently activated by multiple Sec12 molecules recruited to the ER exit sites by cTAGE5 and TANGO1.

Materials and methods

Antibodies

A female 6-wk-old Wistar rat (CLEA Japan) was immunized with FLAG-tagged Sec23A or GST-tagged Sec12 (93–239 aa) in TiterMax Gold (TiterMax USA). Splenocytes were fused with PAI mouse myeloma cells using polyethylene glycol (Roche). Hybridoma supernatants were screened by indirect ELISA with His-tagged Sec23A or ColdTF-tagged Sec12 (93–239 aa) as the antigens. Positive hybridoma lines were subcloned, grown in serum-free medium (Nihon Pharmaceutical) supplemented with hypoxanthine-thymidine (Thermo Fisher Scientific), and purified with protein G-Sepharose (GE Healthcare; Saito et al., 2014). Polyclonal antibodies against Sec16-N (374–387 aa), Sec16-C (2,319–2,332 aa), TANGO1-CT (1,884–1,898 aa), and cTAGE5-CT (791–804 aa) were raised in rabbits by immunization with keyhole limpet hemocyanin-conjugated peptides and affinity-purified by columns conjugated with the peptides (Thermo Fisher Scientific; Iinuma et al., 2007; Saito et al., 2009, 2011; Maeda et al., 2016; Tanabe et al., 2016). Polyclonal antibodies against TANGO1-CC1 or cTAGE5-CC1 were raised in rabbits by immunization with recombinant GST-tagged TANGO1 (1,231–1,340 aa) or cTAGE5 (118–227 aa) and affinity-purified by columns conjugated with ColdTF-tagged TANGO1 (1,231–1,340 aa) or MBP-tagged cTAGE5 (118–227 aa; Saito et al., 2011; Maeda et al., 2016; Tanabe et al., 2016). Other antibodies were as follows: KDEL (mouse; Enzo Life Sciences), β-actin (mouse; Sigma-Aldrich), GM130 (mouse; BD), FLAG (mouse; Sigma-Aldrich), HA (rat; Roche), GST (mouse; Nakalai Tesque), and Sec31 (mouse; BD).

Constructs

Human Sec16A cDNA used in this study is encoded by RefSeq XM_011519257.1 (2,332 aa). N-terminal FLAG-tagged Sec16 domain (1–550, 551–1,100, 1,101–1,600, 1,101–1,400, 1,401–1,600, 1,601–1,890, and 1,891–2,332 aa), Sec23A, and Sec23B were cloned into pCMV5 vectors that were gifts from D. Russell (University of Texas Southwestern Medical Center, Dallas, TX). For FLAG-tagged cTAGE5 rescue constructs, silent mutations (1,827G→A, 1,830A→G, 1,833A→T, 1,836A→G, 1,837T→A, 1,838C→G, 1,839A→C, 1,845T→A, and 1,848T→C) were introduced to achieve siRNA resistance, and the C-terminal 14 aa were truncated so as not to be recognized by the cTAGE5-CT antibody for immunofluorescence experiments (Saito et al., 2014).

Cell culture and transfection

HeLa and 293T cells were cultured in DMEM supplemented with 10% FBS. Lipofectamine RNAi max (Thermo Fisher Scientific) was used for transfecting siRNA. For plasmid transfection, polyethylenimine MAX (Polysciences) or Fugene 6 (Promega) were used. Doxycycline-inducible stable cell lines expressing TANGO1S-GFP were made with the lentivirus system described previously (Shin et al., 2006).

VSVG-transport assay

siRNA-treated HeLa cells were again transfected with siRNAs. 48 h after initial transfection, cells were transfected with VSVG-ts045-GFP and further incubated for 8 h at 37°C. The cells were shifted to 39.5°C for 12 h to retain the protein in the ER. Cells were replated with ice-cold medium and placed on ice for 1 min. Then, the cells were incubated for indicated times before fixation. Fixed cells were processed for immunofluorescence.

In vitro binding assay

GST, GST-tagged TANGO1-coil1 (1,211–1,440 aa), TANGO1-coil2 (1,441–1,650 aa), TANGO1-PRD (1,651–1,907 aa), TANGO1-PRDΔ45 (1,651–1,862 aa), TANGO1-PRDΔ60 (1,651–1,847 aa), or TANGO1-PRDΔ120 (1,651–1,787 aa) were expressed in *Escherichia coli* and purified with glutathione Sepharose (GE Healthcare). GST fusion proteins were conjugated to glutathione Sepharose and incubated with FLAG-Sec16 (1,101–1,600 aa) or FLAG-Sec23A purified from 293T cells. Beads were washed with TBS/0.1% Triton X-100 five times, followed by elution with glutathione.

Immunoprecipitation and Western blotting

The experiments were essentially performed as described previously (Saito et al., 2014). Cells extracted with extraction buffer consisting of 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors were centrifuged at 20,000 *g* for 15 min at 4°C. Cell lysates were immunoprecipitated with FLAG M2 antibodies (Sigma-Aldrich). The beads were washed with TBS/0.1% Triton X-100 five times, followed by elution with DYKDDDK peptide, and processed for sample preparation.

siRNA oligos

Stealth select siRNAs for panTANGO1, TANGO1S, TANGO1L, Sec16, cTAGE5, Sec23A, and Sec23B were purchased from Thermo Fisher Scientific. The oligo sequences used were as follows: panTANGO1 siRNA, 5′-CAGGAAAUCGAAGACUGGAGUAAAU-3′; TANGO1S siRNA, 5′-GAAUUGUCGCUUGCGUUCAGCUGUU-3′; TANGO1L siRNA, 5′-CAACUCAGAGGAAAGUGAUAGUGUA-3′; Sec16 siRNA (3031), 5′-CCGUCCCAUUCUGACAGCCUCGCUU-3′; Sec16 siRNA (5393), 5′-CCCUGCCUAGUUUCCAGGUGUUUAA-3′; cTAGE5 siRNA (1825), 5′-CCGCCAGGACAAUCAUAUCCUGAUU-3′; cTAGE5 siRNA (91), 5′-GACCAGAUUCUAAUCUUUAUGGUUU-3′; Sec23A siRNA (1119), 5′-GGGUGAUUCUUUCAAUACUUCCUUA-3′; Sec23A siRNA (366), 5′-GCGUGGUCCUCAGAUGCCUUUGAUA-3′; Sec23B siRNA (1021), 5′-GCUGCAAAUGGUCACUGCAUUGAUA-3′; and Sec23B siRNA (1923), 5′-CAGCAGCAUUCUAGCUGACAGAAUU-3′. For control siRNA, stealth RNAi siRNA negative control med GC duplex #2 (Thermo Fisher Scientific) was used. The number in the parentheses represents the starting base pair of the target sequence.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously (Saito et al., 2014). For cells fixed with cold methanol, cells grown on coverslips were washed with PBS, fixed with methanol (6 min at −20°C), washed with PBS, and blocked in blocking solution (5% BSA in PBS with 0.1% Triton X-100 for 30 min). For PFA fixation, cells washed with PBS were fixed with PFA (4% in PBS for 10 min at RT), washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 15 min at RT followed by blocking (5% BSA in PBS with 0.1% Triton X-100 for 30 min). After blocking, cells were stained with primary antibody (1 h at RT) followed by incubation with Alexa Fluor–conjugated secondary antibodies (Alexa Fluor 488, Alexa Fluor 568, or Alexa Fluor 647 for 1 h at RT). Images were acquired with confocal laser scanning microscopy (Plan Apochromat 63×/1.40-NA oil-immersion objective lens; LSM 700; ZEISS). The acquired images were processed with Zen 2009 software (ZEISS). All imaging was performed at RT.

Quantification of Pearson's colocalization coefficient

HeLa cells were treated with control, panTANGO1, TANGO1L, TANGO1S, or cTAGE5 siRNAs. After 48 h, cells were fixed and stained with indicated antibodies. Stained cells were analyzed by confocal laser scanning microscopy (Plan Apochromat 63×/1.40-NA oil-immersion objective lens; LSM 700) and processed with Zen 2009.

Intensity scanning and calculating coefficiency were performed by co-localization plugin Coloc2 in Fiji-ImageJ (Schindelin et al., 2012).

FLAG-tagged constructs were transfected 24 h after siRNA treatment. 48 h after initial transfection, cells were fixed and stained with FLAG antibody and other indicated antibodies. Cells positively stained with the FLAG antibody were categorized as FLAG rescue constructs expressed, and the surrounding cells not stained by the FLAG antibody were categorized as nontransfected counterparts.

Rapamycin-dependent redistribution assay

The rapamycin-dependent redistribution assay was essentially performed as described previously (Komatsu et al., 2010). HeLa cells treated with TANGO1L and TANGO1S siRNAs were transfected with Tomm20 (1–33 aa)-GFP-FRAP (2,026–2,114 aa) construct and TANGO1 PRD domain (1,651–1,907 aa)-HA-FKBP (3–108 aa) or TANGO1 PRD Δ120 (1,651–1,787 aa)-HA-FKBP (3–108 aa) constructs. 48 h after initial transfection, cells were supplemented with 200 nM rapamycin or DMSO and incubated for 2 h at 37°C. Cells were fixed with methanol and stained with indicated antibodies.

Online supplemental material

Fig. S1 shows VSVG transport assay in TANGO1-depleted cells at 0- and 30-min time points. Fig. S2 shows by immunoprecipitation analysis that Sec16 ELD is responsible for interaction with TANGO1. Fig. S3 shows purified recombinant proteins used in the in vitro binding assay. Fig. S4 shows specificities and properties of rat monoclonal antibodies for Sec23. Fig. S5 shows that Sec23 depletion does not affect the localization of endogenous TANGO1.

Acknowledgments

We thank the members of Katada laboratory for valuable discussions.

This work is supported in part by research grants from Japan Society for the Promotion of Science (T. Katada and K. Saito).

The authors declare no competing financial interests.

Author contributions: M. Maeda designed and performed research, analyzed data, and wrote the manuscript; T. Katada designed research and wrote the manuscript; and K. Saito designed and performed research, analyzed data, and wrote the manuscript.

Submitted: 14 March 2017
Revised: 11 April 2017
Accepted: 12 April 2017

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