Concentration of Sec12 at ER exit sites via interaction with cTAGE5 is required for collagen export

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Mechanisms for exporting variably sized cargo from the endoplasmic reticulum (ER) using the same machinery remain poorly understood. COPII-coated vesicles, which transport secretory proteins from the ER to the Golgi apparatus, are typically 60–90 nm in diameter. However, collagen, which forms a trimeric structure that is too large to be accommodated by conventional transport vesicles, is also known to be secreted via a COPII-dependent process. In this paper, we show that Sec12, a guanine-nucleotide exchange factor for Sar1 guanosine triphosphatase, is concentrated at ER exit sites and that this concentration of Sec12 is specifically required for the secretion of collagen VII but not other proteins. Furthermore, Sec12 recruitment to ER exit sites is organized by its direct interaction with cTAGE5, a previously characterized collagen cargo receptor component, which functions together with TANGO1 at ER exit sites. These findings suggest that the export of large cargo requires high levels of guanosine triphosphate–bound Sar1 generated by Sec12 localized at ER exit sites.

Introduction

Newly synthesized secretory proteins exit the ER in COPII-coated vesicles (Brandizzi and Barlowe, 2013; Lord et al., 2013; Miller and Schekman, 2013). COPII-coated carrier formation occurs at ER exit sites and is typically initiated by the activation of small GTPase Sar1 by Sec12 (Nakańo and Muramatsu, 1989; Barlowe and Schekman, 1993). After activation of Sar1, the inner coat complex Sec23/24 is recruited with cargo molecules to form a prebudding complex (Yoshihisa et al., 1993; Kuehn et al., 1998; Miller et al., 2002; Tabata et al., 2009), and binding of the outer coat complex Sec13/31 completes the coat assembly (Stagg et al., 2006; Bi et al., 2007). Another essential protein at ER exit sites is Sec16, which is a scaffold protein that interacts with some coat proteins (Connerly et al., 2005; Watson et al., 2006; Iinuma et al., 2007; Ivan et al., 2008). Sec16 is also thought to act as a negative regulator of GTP hydrolysis by Sar1, inhibiting the recruitment of Sec31 to the prebudding complex (Kung et al., 2012; Yorimitsu and Sato, 2012; Bharucha et al., 2013). COPII vesicles are thought to be cuboctahedral structures with a diameter of 60–90 nm (Stagg et al., 2006). However, some cargo molecules, including collagens, are too large to be accommodated by these structures (Fromme and Schekman, 2005; Malhotra and Erlmann, 2011; Miller and Schekman, 2013).

Emerging evidence suggests that the exit of collagen from the ER occurs via the modification of the conventional COPII-mediated export system through the help of specific dedicated proteins. We previously identified mammalian TANGO1 and its interactor, cTAGE5, as a cargo receptor for collagen VII at ER exit sites. Specifically, the luminal SH3 domain of TANGO1 interacts with collagen VII, and cytoplasmic proline-rich domains of both cTAGE5 and TANGO1 interact with the Sec23/24 complex (Saito et al., 2009, 2011). We proposed that the interaction of cTAGE5–TANGO1 with Sec23/24 delays the recruitment of Sec13/31, thereby modifying the conventional COPII coat formation to accommodate large carriers. Recently, collagen secretion was also found to involve the interaction of TANGO1 with Sedlin, a member of the transport protein particle complex required for ER-to-Golgi tethering (Venditti et al., 2012), and SLY1, a protein required for membrane fusion (Nogueira et al., 2014). Another example of transport system

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Abbreviations used in this paper: BFA, Brefeldin A; CT, C terminus; ERGIC, ER–Golgi intermediate compartment; GEF, guanine-nucleotide exchange factor; MBP, maltose-binding protein; qRT-PCR, quantitative RT-PCR; RT-PCR, real-time PCR.

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modulation is the identification of Sec31 monoubiquitylation by the CUL3-KLHL12 system for large transport carrier formation (Jin et al., 2012). Note that the studies on collagen transport have also revealed the mechanisms for intra-Golgi trafficking. The cisternae progression–maturation model was initially confirmed by electron microscopic analysis of collagen trafficking inside the Golgi in mammalian cells (Bonfanti et al., 1998; Glick and Luini, 2011). Recently, it has been reported that soluble cargoes can traverse through cisternae more quickly than collagen by diffusion-based mechanism (Beznoussenko et al., 2014).

Sec12 is a type II transmembrane protein containing seven WD-40 folds that is conserved across species. Although mammalian Sec12 has been isolated and described as a guanine-nucleotide exchange factor (GEF) for Sar1 (Weissman et al., 2001), its characterization has been limited to date. Here, we show that Sec12 is concentrated to the ER exit sites in mammalian cells. Interestingly, Sec12 localization at ER exit sites is specifically required for collagen exit from the ER but not for general protein secretion. Furthermore, cTAGE5, a previously characterized collagen coreceptor of TANGO1, is responsible for the recruitment of Sec12 to ER exit sites. These results suggest that large cargo may require higher levels of activated Sar1 concentrated at ER exit sites, which could be achieved via localized Sec12.

Results

cTAGE5 interacts with Sec12 at ER exit sites

To further characterize the role of cTAGE5 in collagen secretion, we searched for cTAGE5-binding proteins by immunoprecipitating endogenous cTAGE5 from HeLa cell extracts. As previously described (Saito et al., 2011), cTAGE5 efficiently coimmunoprecipitated TANGO1. In addition, an ∼45-kD protein was revealed as a coimmunoprecipitant of cTAGE5 by silver staining (Fig. 1 A) and was identified as Sec12 by matrix-assisted laser desorption/ionization–time of flight mass spectrometry analysis.

Rat monoclonal antibodies were produced against Sec12, and their specificities were checked using Sec12 knockdown cells. Among them, clone 7A10 specifically recognized Sec12 in Western blotting (see Fig. 3 F), and clone 6B3 was suitable for immunoprecipitation (Fig. 1 C, bottom). Western blotting of cTAGE5 immunoprecipitants with Sec12 antibody (clone 7A10) revealed the band corresponding to 45 kD (Fig. 1 A, bottom). Knockdown of cTAGE5 before immunoprecipitation with cTAGE5 antibody reduced cTAGE5, TANGO1, and Sec12 signals, indicating the specificity of the interaction (Fig. 1 B). Moreover, Sec12 antibody (clone 6B3) and other clones efficiently coimmunoprecipitated cTAGE5 (Figs. 1 C and S1 A). Therefore, Sec12 was found to interact with cTAGE5.

Previous observations indicate that Sec12 is dispersed throughout the ER (Weissman et al., 2001), although Montegna et al. (2012) recently found punctate staining of Sec12 that colocalized with a marker of ER exit sites, in addition to the reticular ER. To see whether Sec12 localizes to the ER exit sites where cTAGE5 has been shown to locate, we performed immunofluorescence analysis with monoclonal antibodies. By fixing with methanol, a suitable method for staining ER exit sites, we found that Sec12 was concentrated to punctate structures and substantially colocalized with proteins at ER exit sites, such as Sec16, cTAGE5, and TANGO1 (Fig. 1 D). The Sec12 signal was diminished by either preincubation with recombinant Sec12 (Fig. S1 B) or knockdown of Sec12 (see Fig. 3 A), indicating its specificity. Brefeldin A (BFA) treatment did not affect Sec12 localization (Fig. S1 C). Thus, we found that Sec12 is concentrated to the ER exit sites and interacts with cTAGE5.

cTAGE5 does not affect guanine-nucleotide exchange activity of Sec12 toward Sar1

As Sec12 is a GEF for Sar1, we investigated whether the interaction between cTAGE5 and Sec12 changes the catalytic activity of Sec12 toward Sar1. We preloaded Sar1 with [^{3}H]GDP and measured the dissociation rate of the nucleotide in the presence of proteins purified from baculovirus-infected Sf9 cells (Fig. 2 A). Incubation with larger amounts of Sec12 significantly enhanced the dissociation rates, confirming that Sec12 acts as a GEF for Sar1 (Fig. 2 B). The addition of cTAGE5, together with or without TANGO1, however, did not change the dissociation rate (Fig. 2, C and D). Thus, the interaction with cTAGE5 does not exert its influence on the GEF activity of Sec12.

Sec12 delocalizes from the ER exit sites upon cTAGE5 depletion

We examined the localization of Sec12 in cTAGE5-depleted cells. With methanol fixation, knockdown of cTAGE5 severely reduced the presence of Sec12 at ER exit sites, although Sec16 remained present at punctate structures characteristic of ER exit sites (Fig. 3 A). In contrast, knockdown of Sec12 had little effect on the localization of either cTAGE5 or Sec16 (Fig. 3 A). Of note, knockdown of TANGO1 mildly affected the localization of Sec12 (Fig. S2 A) as well as that of cTAGE5, as previously reported (Saito et al., 2011). We next examined the localization of Sec12 in PFA-fixed cells. Upon cTAGE5 knockdown, the punctate staining of Sec12 is reduced but still faintly observed throughout the cells (Fig. 3 B). However, the substantial colocalization of Sec12 with Sec16 in control cells has markedly decreased in cTAGE5-depleted cells (Fig. 3, B and C). It seemed not merely the result of signal reduction, but rather, Sec12 has changed its localization from the ER exit sites (Fig. 3 B, right). We then checked the possibility that Sec12 shifted the localization to the ER–Golgi intermediate compartment (ERGIC) or reticular ER by costaining with ERGIC-53 or KDEL. The colocalization of Sec12 with both markers was limited and even decreased upon cTAGE5 knockdown (Fig. 3, D and E; and Fig. S2, B and C), implying that Sec12 is not mainly localized to these organelles in cTAGE5-depleted cells. We investigated the protein content of Sec12 in cTAGE5-depleted cells. Although Sec12 staining seemed to be reduced by cTAGE5 knockdown (Fig. 3, A and B), Western blotting of corresponding cell lysates revealed that Sec12 protein expression level was unchanged (Fig. 3 F). To further explore the Sec12 localization in cTAGE5-depleted cells, we separated cellular organelles from control and cTAGE5 siRNA-treated cells using density gradient centrifugation. In control cells, Sec12

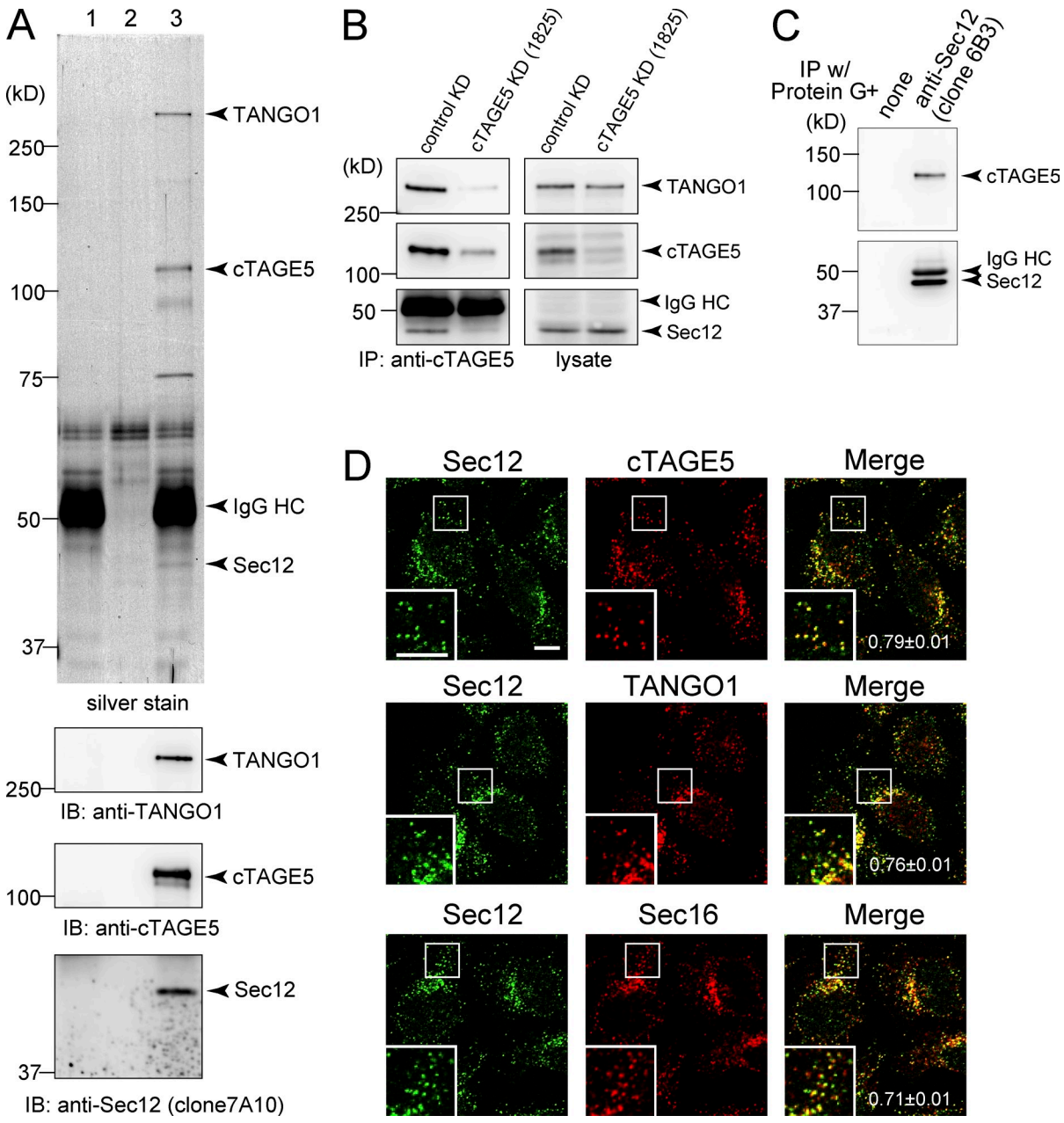

Figure 1. **cTAGE5 interacts with Sec12 at ER exit sites.** (A) Protein A beads conjugated with (lanes 1 and 3) or without (lane 2) cTAGE5 CC1 antibody were incubated with (lanes 2 and 3) or without (lane 1) HeLa cell lysates. Beads were washed, and proteins retained by beads were analyzed by SDS-PAGE followed by silver staining or Western blotting with TANGO1, cTAGE5 CC1, and Sec12 (clone 7A10) antibodies. HC, heavy chain. (B) HeLa cells were transfected with control or cTAGE5 siRNA (1,825). After 48 h, cell lysates were prepared and immunoprecipitated with the cTAGE5 CC1 antibody conjugated to protein A beads. Cell lysates and immunoprecipitants were resolved on SDS-PAGE and subject to Western blotting with TANGO1, cTAGE5 CC1, and Sec12 (clone 7A10) antibodies. (C) Protein G beads were untreated or conjugated with Sec12 antibody (clone 6B3) and then incubated with HeLa cell lysates. Beads were washed, and proteins retained by beads were analyzed by SDS-PAGE followed by Western blotting with cTAGE5 CC1 and Sec12 (clone 7A10) antibodies. (D) HeLa cells fixed with cold methanol were subject to staining with Sec12 (clone 6B3) antibody and cTAGE5 CT, TANGO1, or Sec16 antibody. Insets show magnification of the indicated regions. The Mander's colocalization coefficient of Sec12 and cTAGE5, TANGO1, or Sec16 is shown in the bottom of the merged insets. $n = 6$. Means ± SEM. IB, immunoblot; IP, immunoprecipitation. Bars, 5 µm.

cofractionated with the ER marker protein calnexin and separated with the early endosome marker EEA1 and the Golgi marker GM130 (Fig. 3 G, top). This pattern of fractionation was not significantly changed by cTAGE5 knockdown (Fig. 3 G, bottom), suggesting that Sec12 is at least preserved at some membranous structures fractionated as the ER even after cTAGE5 knockdown.

Sec12 localizes to the ER exit sites via interaction with cTAGE5

cTAGE5 contains two coiled-coil domains and a proline-rich domain (Fig. 4 C). To elucidate the interaction domain in cTAGE5, we coexpressed FLAG-tagged cTAGE5 deletion mutants with HA-tagged Sec12 cytoplasmic domains in 293T cells. Cell lysates were immunoprecipitated with anti-FLAG antibodies followed by

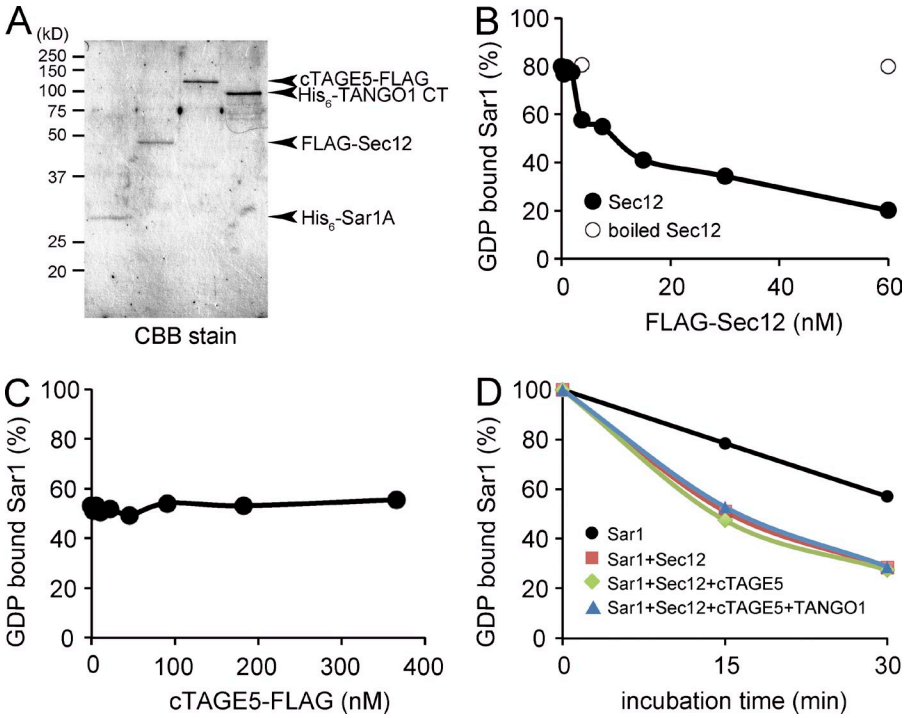

Figure 2. **Interaction between cTAGE5 and Sec12 does not affect guanine-nucleotide exchange activity of Sec12 toward Sar1.** (A) Coomassie brilliant blue (CBB) staining of proteins purified from baculovirus-infected Sf9 cells. (B) 100 nM Sar1 was preloaded with [^{3}H]GDP and then incubated with the indicated concentrations of Sec12 for 30 min at 30°C. The amount of [^{3}H]GDP bound to Sar1 was quantified. The experiments were repeated twice. For the experiments shown, $n = 3$. (C) 100 nM Sar1 was preloaded with [^{3}H]GDP and then incubated with 15 nM Sec12 and the indicated concentrations of cTAGE5 for 15 min at 30°C. The experiments were repeated twice. The data shown are from a single representative experiment. For the experiment shown, $n = 3$. (D) 100 nM Sar1 was preloaded with [^{3}H]GDP and then incubated with or without 15 nM Sec12, 100 nM cTAGE5, and 100 nM TANGO1 CT for the indicated times at 30°C. The experiments were repeated twice. The data shown are from a single representative experiment. For the experiment shown, $n = 4$.

elution with the FLAG peptide. We found that the coil1 region of cTAGE5 interacted specifically with Sec12 (Fig. 4 A). To test whether this interaction is direct, we created recombinant proteins of coiled-coil regions fused with maltose-binding protein (MBP) and assessed their interaction with Sec12 purified from Sf9 cells. We found that cTAGE5 interacted directly with Sec12 (Fig. 4 B), with both the coil1 and coil2 regions of cTAGE5 having a capacity to interact with Sec12. In contrast, neither coil region of TANGO1 interacted with Sec12. These findings suggest that Sec12 directly binds to cTAGE5 but not to TANGO1.

Next, by generating FLAG-tagged siRNA-resistant cTAGE5 constructs, we tested whether localization of Sec12 to ER exit sites is directly regulated by its interaction with cTAGE5. One cTAGE5 construct, referred to as cTAGE5–[coil1T1], had its coil1 region swapped with that of TANGO1 (Fig. 4 C). Both control cTAGE5-FLAG and cTAGE5–[coil1T1]-FLAG rescue constructs localized at ER exit sites when expressed in cTAGE5 knockdown cells (Fig. 4 E). As expected, control cTAGE5-FLAG interacted with both Sec12 and TANGO1 in 293T cells (Fig. 4 D). cTAGE5–[coil1T1]-FLAG also interacted with TANGO1 but did not bind to Sec12. Thus, cTAGE5–[coil1T1] appeared to be capable of forming cTAGE5–TANGO1 but not cTAGE5–Sec12 complexes at ER exit sites. The expression of cTAGE5-FLAG in cTAGE5-depleted cells efficiently rescued the localization of Sec12 to ER exit sites (Fig. 4 E). However, cTAGE5–[coil1T1]-FLAG, which did not bind to Sec12, failed to recruit Sec12 to ER exit sites (Fig. 4 E). These results strongly suggest that Sec12 localization to ER exit sites is directly controlled by its interaction with cTAGE5.

Sec12 localization is regulated by different mechanism between *Pichia pastoris* and mammalian cells

Previous studies suggest that in *P. pastoris*, Sec16 directly binds to Sec12 and recruits Sec12 to ER exit sites (Soderholm et al., 2004; Montegna et al., 2012). Specifically, Sec12 overexpression in *P. pastoris* delocalizes Sec12 to the ER network, whereas Sec16 coexpression retains Sec12 at ER exit sites. As human Sec16 also binds to Sec12, this mechanism might be conserved across species. Our present results suggest that Sec12 is recruited to ER exit sites by interacting with cTAGE5 in mammalian cells. To check whether cTAGE5- and Sec16-mediated localization of Sec12 to ER exit sites is cooperative or independent, we examined Sec12 localization after Sec16 overexpression in cTAGE5 knockdown cells. Knockdown of cTAGE5 led to the dispersion of Sec12 without changing the localization of Sec16 (Fig. 3 A). Simultaneous overexpression of Sec16 failed to rescue its localization at ER exit sites, in spite of correct localization of overexpressed Sec16 to the ER exit sites (Fig. 4 F). Therefore, Sec16 and cTAGE5 are independently required for Sec12 localization to ER exit sites. We also attempted to examine the localization of Sec12 after Sec16 knockdown and cTAGE5 overexpression, but this experiment was not possible because knockdown of Sec16 caused the dispersion of not only Sec12 but also cTAGE5, consistent with the idea that Sec16 is an upstream organizer of ER exit sites (Fig. S3).

cTAGE5-mediated Sec12 localization to ER exit sites is required for collagen VII secretion but not for general protein secretion

To clarify the functional significance of Sec12 recruitment to ER exit sites by cTAGE5, we tested its impact on protein secretion. We previously reported that cTAGE5 knockdown causes accumulation of collagen VII within the ER (Saito et al., 2011). Real-time PCR (RT-PCR) confirmed that knockdown of cTAGE5 did not increase collagen VII mRNA levels, suggesting that the accumulated collagen VII within the ER is not caused by excessive synthesis but by transport block from the ER (Fig. S4). Next, transport of mannosidase II from the ER to

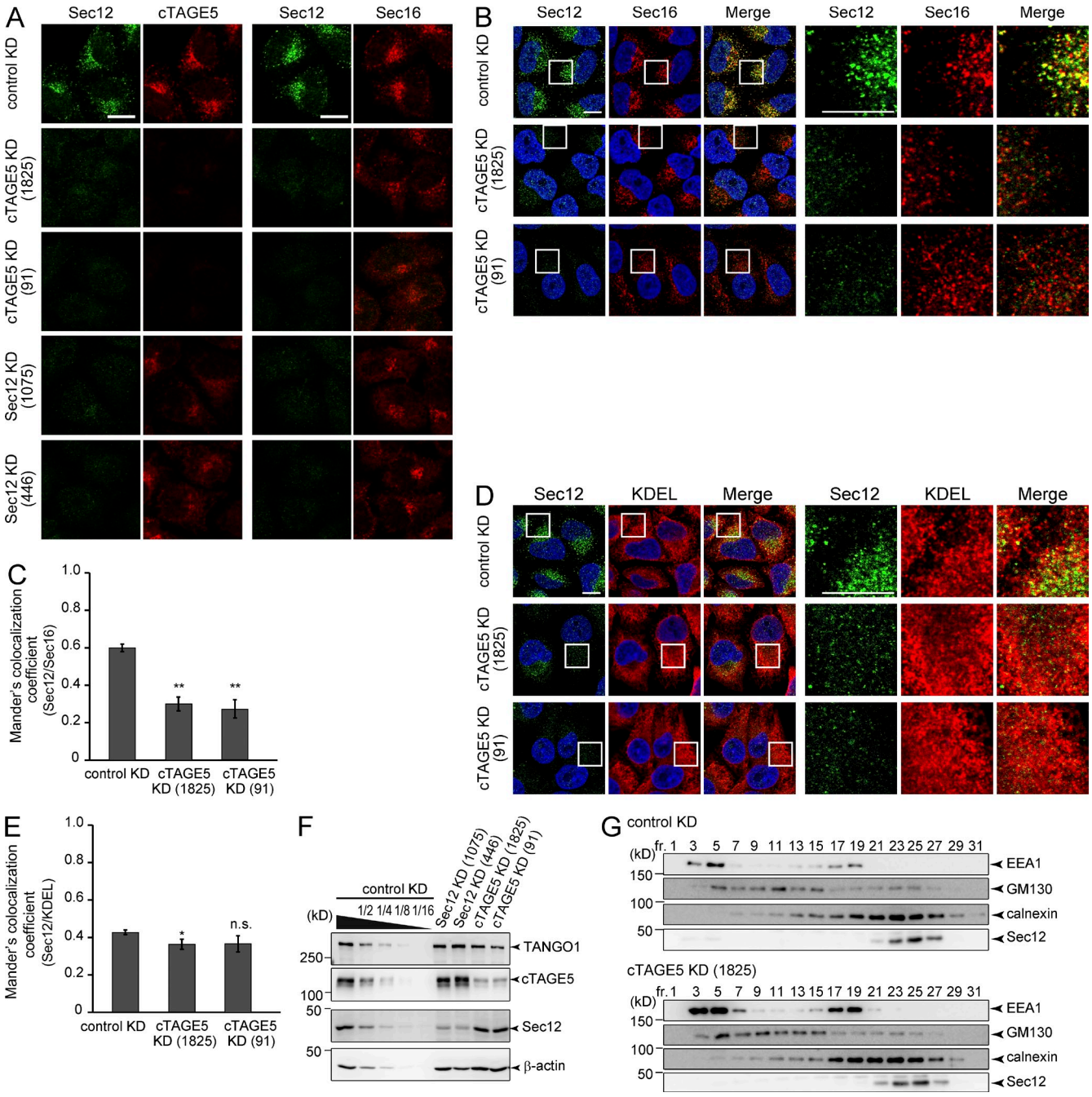

Figure 3. **Sec12 is dispersed from the ER exit sites upon cTAGE5 knockdown.** (A) HeLa cells were transfected with control, cTAGE5, or Sec12 siRNA. After 48 h, the cells were fixed with cold methanol and stained with Sec12, cTAGE5 CT, or Sec16 antibodies. (B) HeLa cells were transfected with control or cTAGE5 siRNA. After 48 h, cells were fixed with PFA and stained with Sec12 and Sec16 antibodies. The nuclei were stained with DAPI (blue). (right) Magnifications of the indicated regions on the left. (C) Quantification of Mander's colocalization coefficient of B. $n = 4$. Error bars represent means ± SEM. **, $P < 0.001$. (D) HeLa cells were transfected with control or cTAGE5 siRNA. After 48 h, cells were fixed with PFA and stained with Sec12 and KDEL antibodies. The nuclei were stained with DAPI (blue). (right) Magnifications of the indicated regions on the left. (E) Quantification of Mander's colocalization coefficient of D. $n = 4$. Error bars represent means ± SEM. *, $P < 0.05$. (F) HeLa cells transfected with siRNAs were extracted and subject to SDS-PAGE followed by Western blotting with TANGO1, cTAGE5 CC1, Sec12 (clone 7A10), and β-actin antibodies. For control knockdown, lysates were serially diluted. (G) HeLa cells were treated with control or cTAGE5 siRNA. After 48 h, HeLa cells were collected, and postnuclear supernatants were fractionated using a 0–26% OptiPrep density gradient. Fractions (fr.) were collected and analyzed by SDS-PAGE followed by Western blotting with EEA1, GM130, calnexin, and Sec12 (clone 7A10) antibodies. KD, knockdown. Bars, 10 µm.

Golgi after BFA removal was investigated in cells depleted of cTAGE5 and Sec12. Although cTAGE5 knockdown induced some Golgi dispersion as shown by mannosidase II staining after BFA recovery and GM130 (Figs. 5 A and S5), transport itself was not affected. In contrast, Sec12 knockdown severely delayed mannosidase II transport (Fig. 5 A). Although we have previously shown that cTAGE5 knockdown did not alter secretion of most of the cargoes by investigating proteins secreted to the medium, the rate of transport was not precisely measured at that point (Saito et al., 2011). For further validation, the cells

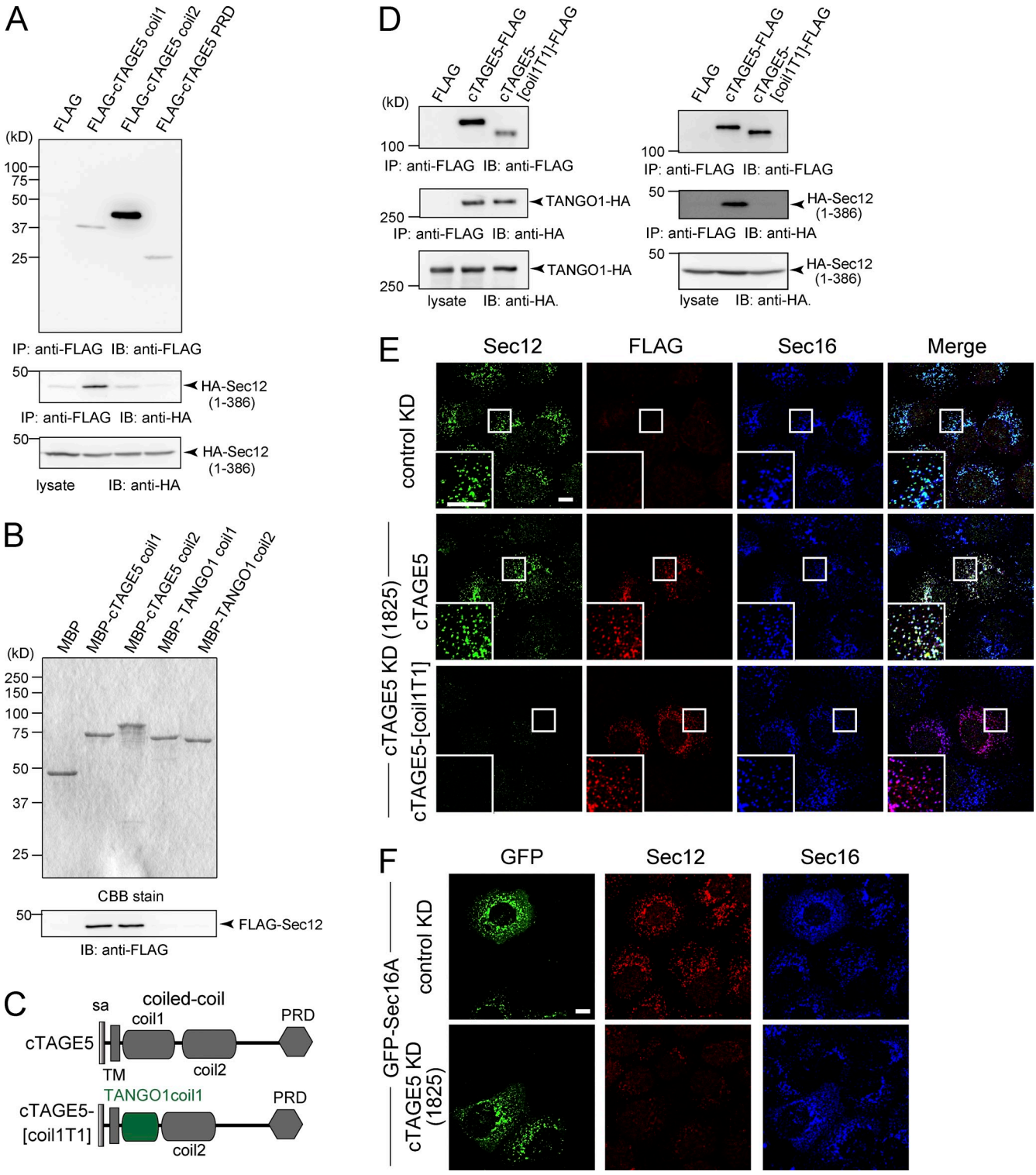

Figure 4. **Interaction with cTAGE5 is necessary for localization of Sec12 at ER exit sites.** (A) 293T cells were transfected with FLAG-tagged cTAGE5 coil1 (61–300 aa), FLAG-tagged cTAGE5 coil2 (301–650 aa), FLAG-tagged cTAGE5 proline-rich domain (PRD; 651–804 aa), or HA-tagged Sec12 (1–386 aa). The cell lysates were immunoprecipitated with anti-FLAG antibody and eluted with the FLAG peptide. Eluates and cell lysates were analyzed by SDS-PAGE followed by Western blotting with FLAG or HA antibodies. (B) MBP or MBP-tagged cTAGE5-coil1, cTAGE5-coil2, TANGO1-coil1, or TANGO1-coil2 were expressed in *E. coli* and purified with amylose resin. (top) Purified proteins were analyzed by SDS-PAGE followed by Coomassie brilliant blue (CBB) staining. FLAG-Sec12 was purified from baculovirus-infected Sf9 cells. MBP and MBP-tagged cTAGE5 or TANGO1 coil domain structures were immobilized to amylose resin and incubated with FLAG-Sec12. Resins were washed and eluted with maltose. (bottom) Eluted proteins were subject to SDS-PAGE followed by Western blotting with the FLAG antibody. (C) Domain organization of cTAGE5 mutant constructs. sa, signal anchor; TM, transmembrane. (D) 293T cells were transfected with cTAGE5-FLAG constructs or cTAGE5–[coil1T1]-FLAG with HA-tagged TANGO1 or Sec12 (1–386 aa). Cell lysates were immunoprecipitated with the FLAG antibody and eluted with the FLAG peptide. Eluates and cell lysates were analyzed by SDS-PAGE followed by Western blotting with FLAG or HA antibodies. (E) HSC-1 cells were transfected with control or cTAGE5 siRNA (1,825) and cultured for 24 h. cTAGE5-FLAG

transfected with cTAGE5 siRNA were 1-h pulse-cultured with [^{35}S]methionine, and the medium was collected at 0, 30, 60, and 90 min to see the time-dependent secretion of radiolabeled proteins. The proteins secreted to the medium were increased over time; however, there were no significant differences between control and knockdown cells even in the early phase (Fig. 5 B), indicating that the rate of secretion was intact for most of the proteins in cTAGE5-depleted cells. Thus, cTAGE5 is specifically required for collagen VII secretion but not for general protein secretion. Next, we examined collagen secretion under Sec12-depleted conditions. As expected, Sec12 knockdown caused an accumulation of collagen VII in the ER (Fig. 5 C) similar to that observed after cTAGE5 knockdown (Saito et al., 2011), suggesting that both cTAGE5 and Sec12 are required for collagen VII exit from the ER. Finally, we tested the effect of cTAGE5 rescue constructs on collagen secretion. When control cTAGE5-FLAG was expressed in cTAGE5-depleted cells, there was significantly less collagen VII accumulation compared with that in nontransfected cells (Fig. 5 D). In contrast, expression of cTAGE5–[coil1T1]-FLAG, which failed to rescue Sec12 localization at ER exit sites, did not affect the accumulation of collagen VII. These results strongly suggest that Sec12 recruitment to ER exit sites by cTAGE5 is important for the exit of collagen VII from the ER.

cTAGE5-mediated Sec12 concentration to the ER exit sites is not regulated by collagen secretion

To check whether the amount of collagens to be secreted regulates the cTAGE5-mediated Sec12 concentration to the ER exit sites, we investigated the localization of Sec12 and cTAGE5 under the conditions that collagen secretion was impaired. We have treated HSC-1 cells with collagen VII siRNA and analyzed the localization of Sec12 and cTAGE5. As shown in Fig. 6 A, Sec12 and cTAGE5 are still localized at punctate structures characteristic of ER exit sites in collagen VII–depleted cells. We then incubated the cells with dipyridyl, an iron chelator, which inhibits hydroxylation and secretion of broader ranges of collagens (Mironov et al., 2003). Incubation with dipyridyl did not affect the localization of Sec12 as well as cTAGE5 (Fig. 6 B), suggesting that inhibition of collagen secretion did not influence on the localization of Sec12. We also checked the expression level of cTAGE5 and Sec12 on various cell lines, including suspension cells such as Jurkat and Raji, which express fewer collagens if any. cTAGE5, as well as Sec12, is significantly expressed in all cell lines tested (Fig. 6 C), indicating that cTAGE5 expression is not correlated with the amount of collagens secreted. In addition, Sec12 is also concentrated to the ER exit sites and partially colocalized with cTAGE5 in suspension cell lines (Fig. 6 D), suggesting that cTAGE5-mediated Sec12 localization to the ER exit sites is required for but not organized by collagen secretion.

Discussion

In this study, we showed that Sec12 recruitment to ER exit sites by interactions with cTAGE5 is necessary for collagen transport from the ER. As the interaction between cTAGE5 and Sec12 did not alter the activity of Sec12 toward Sar1, cTAGE5-mediated Sec12 concentration may be important for the localized activation of Sar1 in the vicinity of the ER exit sites. In this regard, it has been reported that Sedlin is somehow involved in the inactivation of Sar1 through interaction with TANGO1 at ER exit sites and is specifically required for collagen secretion (Venditti et al., 2012). Thus, it is interesting to speculate that the exit of collagen from the ER may rely on specific activation–inactivation mechanisms—Sar1 might first be activated by the cTAGE5–Sec12 complex and then inactivated by TANGO1–Sedlin. In this case, tight regulation of the Sar1 GTPase cycle at ER exit sites would be important for collagen secretion from the ER, although we cannot totally rule out the possibility that concentrated Sec12 may function other than the activation of Sar1 at ER exit sites.

Together with our finding that cTAGE5 knockdown has little effect on general protein secretion (Fig. 5 B; Saito et al., 2011), the present finding that cTAGE5 knockdown leads to Sec12 delocalization from the ER exit sites suggests that most cargo may exit the ER without the concentration of Sec12 at ER exit sites. A more probable explanation is that the exit of each type of cargo from the ER may require a different amount of activated Sar1, with collagen being a particularly demanding cargo molecule. Other types of cargo, however, may require only a minimal amount of Sec12 at ER exit sites, even in cTAGE5-depleted cells. In this respect, organization of ER exit sites in cTAGE5-depleted cells might resemble the situation in *Saccharomyces cerevisiae*, in which large cargo secretion is probably not required and Sec12 is scattered throughout the ER network, whereas COPII-coated proteins and Sec16 show a punctate organization characteristic of ER exit sites (Okamoto et al., 2012).

Another, more drastic possibility is that ER exit sites in mammalian cells could be specialized domains working only to secrete large cargo. If so, general proteins might be secreted from the ER independent of exit sites in certain conditions. Then, conventional COPII vesicles might form and bud from locations within the ER network other than exit sites. Alternatively, proteins might exit the ER via a COPII-independent pathway. The latter situation has been recently reported in Sar1-depleted cells that procollagen secretion has been impaired without affecting the secretion of other proteins (Cutrona et al., 2013). The authors claimed that a COPII-independent, possibly COPI-dependent, system exists as a substitute pathway for secretion in Sar1-deficient cells. Although it is difficult to draw conclusions at this stage, cells deprived of cTAGE5 might adopt one of these possible mechanisms.

constructs or cTAGE5–[coil1T1]-FLAG were then transfected, and the cells were cultured for 24 h, fixed, and stained with Sec12 (clone 6B3), FLAG, and Sec16 antibodies. Most cTAGE5-FLAG–transfected cells (96/99) were rescued with Sec12 localization to the ER exit sites, in contrast to the few cells (only 1/71) that were rescued with cTAGE5–[coil1T1]-FLAG transfection. Insets show magnification of the indicated regions. (F) HSC-1 cells transfected with control or cTAGE5 siRNA (1,825) were transfected with GFP-Sec16A. Cells were fixed and stained with GFP, Sec12 (clone 6B3), and Sec16 antibodies. IB, immunoblot; IP, immunoprecipitation; KD, knockdown. Bars, 10 µm.

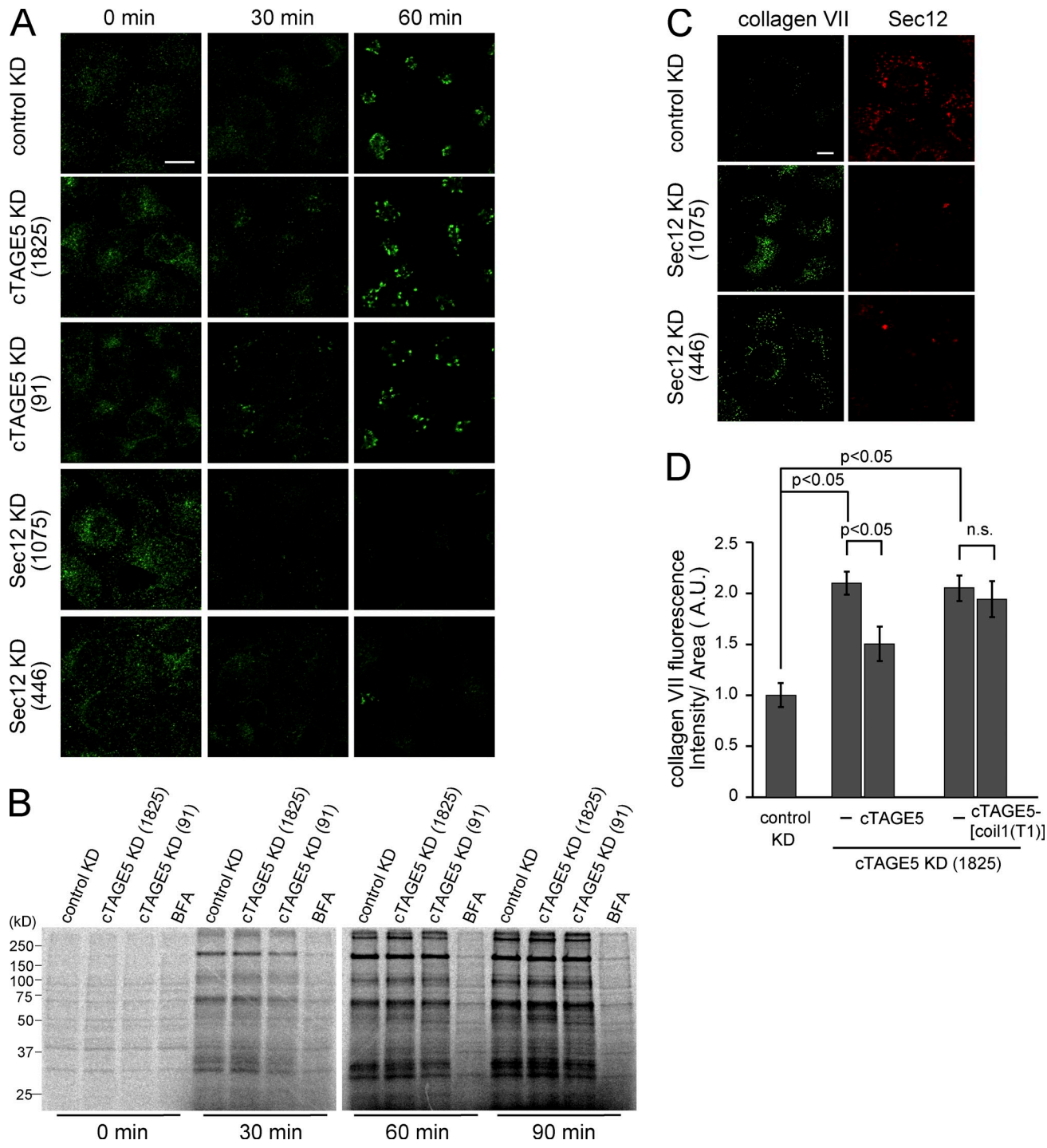

Figure 5. **Localization of Sec12 to ER exit sites is necessary for collagen VII secretion.** (A) HeLa cells were treated with control, cTAGE5, or Sec12 siRNA. After 60 h, the cells were incubated with 5 µg/ml BFA for 30 min at 37°C, washed five times with PBS, and then incubated for the indicated times with 100 µg/ml cycloheximide. Cells were fixed and stained with the mannosidase II antibody. (B) HeLa cells were transfected with control or cTAGE5 siRNA. After 48 h, the cells were labeled with [^{35}S]methionine for 1 h. The cells were washed and then cultured in medium containing unlabeled methionine. At the indicated times (0, 30, 60, and 90 min) after the chase, the medium was collected and analyzed by SDS-PAGE followed by autoradiography. BFA was added for the last 10 min of incubation with labeled methionine and kept throughout the chase. (C) HSC-1 cells were transfected with control, Sec12 (1,075), or Sec12 (446) siRNA. After 48 h, the cells were fixed with cold methanol and stained with collagen VII and Sec12 (clone 6B3) antibodies. (D) HSC-1 cells were treated with control or cTAGE5 siRNA and cultured for 24 h. For control siRNA-treated cells, FLAG-mock was transfected and further cultured for 24 h. For cTAGE5 siRNA–treated cells, cTAGE5-FLAG or cTAGE5–[coil1T1]-FLAG were transfected and further cultured for 24 h. The cells were fixed and stained with collagen VII and FLAG antibodies. Collagen VII immunofluorescence signal per cell (A.U., arbitrary units) was quantified in each cell category described at the bottom. For cells treated with control siRNA, $n = 40$. For cells treated with cTAGE5 siRNA and cTAGE5–FLAG is not expressed, $n = 160$. For cells treated with cTAGE5 siRNA and cTAGE5–FLAG is expressed, $n = 40$. For cells treated with cTAGE5 siRNA and cTAGE5–[coil1T1]-FLAG is not expressed, $n = 125$. For cells treated with cTAGE5 siRNA and cTAGE5–[coil1T1]-FLAG is expressed, $n = 40$ (analysis of variance). Means ± SEM. The data shown are from a single representative experiment out of three repeats. KD, knockdown. Bars, 10 µm.

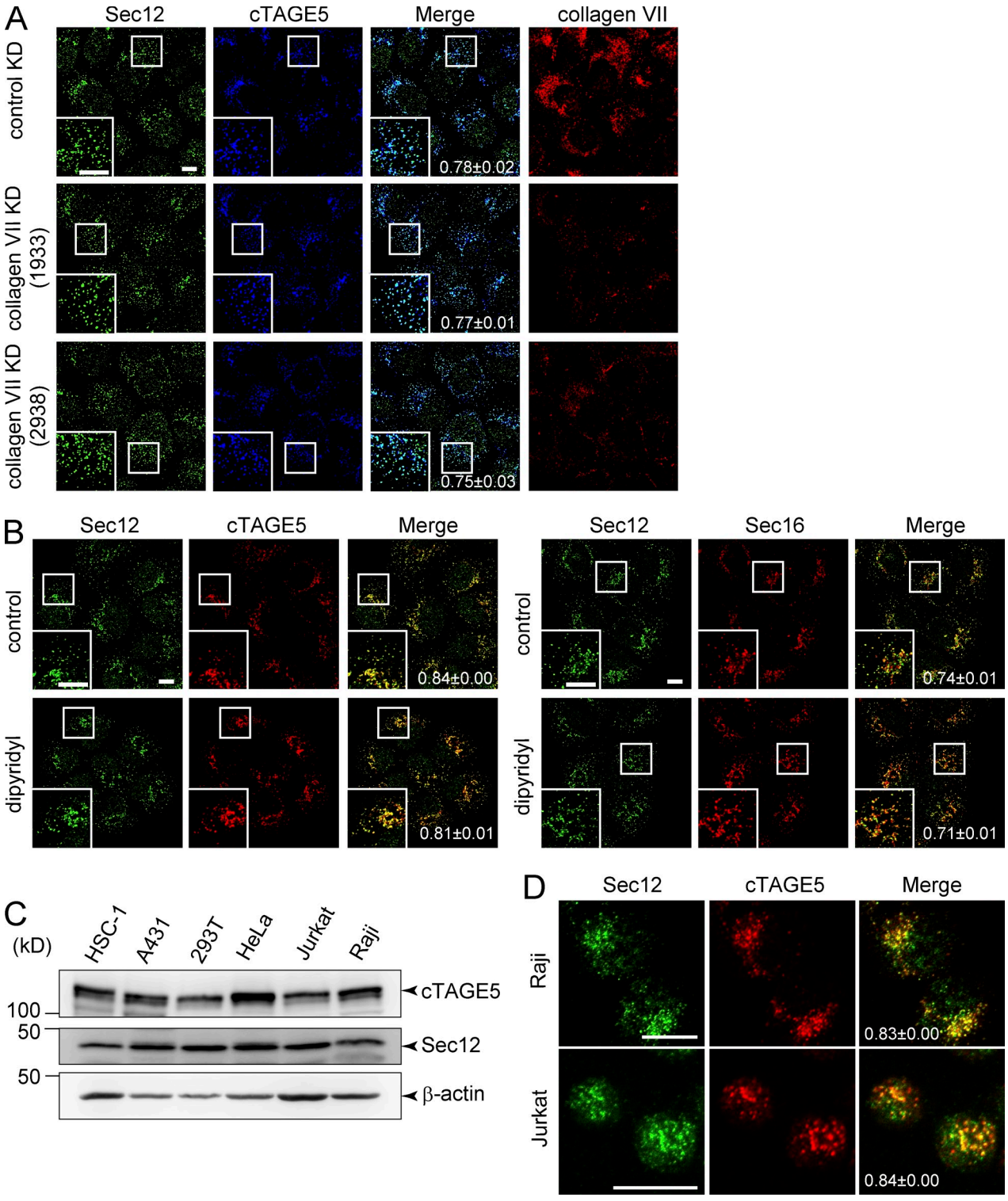

Figure 6. **Sec12 is concentrated to ER exit sites irrespective of collagen secretion.** (A) HSC-1 cells were treated with control or collagen VII siRNA. After 48 h, cells were fixed with cold methanol and stained with Sec12, cTAGE5 CT, and collagen VII antibodies. Insets show magnification of the indicated regions. The Mander's colocalization coefficient of Sec12 and cTAGE5 is shown in the bottom of the merged insets. $n = 5–10$. Means ± SEM. KD, knockdown. (B) HSC-1 cells were incubated with or without 0.6 mM dipyridyl for 1 h at 37°C. Treated cells were fixed with cold methanol and stained with Sec12, cTAGE5 CT, or Sec16 antibodies. Insets show magnifications of the indicated regions. The Mander's colocalization coefficient of Sec12 and cTAGE5 or Sec16 is shown in the bottom of the merged insets. $n = 5–12$. Means ± SEM. (C) Cell lysates prepared from HSC-1, A431, 293T, HeLa, Jurkat, and Raji cells were resolved on SDS-PAGE and subject to Western blotting with cTAGE5 CC1, Sec12 (clone 7A10), and β-actin antibodies. (D) Raji and Jurkat cells were cultured for 30 min on poly-L-lysine–coated coverslips. The cells were fixed with cold methanol and stained with Sec12 and cTAGE5 CT antibodies. The Mander's colocalization coefficient of Sec12 and cTAGE5 is shown in the bottom of the merged insets. $n = 4$. Means ± SEM. Bars, 10 μm.

We showed that Sec12 delocalizes from the ER exit sites in cTAGE5-depleted cells. Although the amount of Sec12 expressed was unchanged, the signal of Sec12 seemed reduced both under methanol- and PFA-fixed conditions and showed very limited colocalization with ERGIC and ER markers by immunofluorescence. Biochemical experiments revealed that Sec12 still localizes to the some membranous fractions, which co-migrate with ER by density gradient fractionation. It is very difficult to conclude where Sec12 relocalized from the ER exit sites, one possible explanation of signal reduction upon cTAGE5 depletion is that Sec12 might be largely diffused from the ER exit site to certain membranous structures related to the ER, such as COPII-dependent vesicular fractions or vast reticular ER. Further investigation is required for tracking down the exact location of Sec12 in cTAGE5-depleted cells. Nonetheless, the present study suggests that cTAGE5-mediated Sec12 localization to the ER exit sites is necessary for collagen export from the ER.

We previously showed that TANGO1 knockdown causes the dispersion of cTAGE5 (Saito et al., 2011). Accordingly, the diffusion of Sec12 upon TANGO1 depletion observed in the present study was likely caused by an indirect effect via the loss of cTAGE5 from ER exit sites. Likewise, Sec16 knockdown led to the dispersion of cTAGE5 as well as Sec12. On the other hand, cTAGE5 knockdown had no appreciable effects on the localization of Sec16. These results make it difficult to quantify the contribution of Sec16 and cTAGE5 to the recruitment of Sec12 to ER exit sites. Nevertheless, our results strongly suggest that an interaction with Sec16 is not sufficient for Sec12 to localize to ER exit sites.

We have previously shown that coil2 regions of both cTAGE5 and TANGO1 are responsible for the interaction between these two proteins. This is confirmed by the present finding that cTAGE5–[coil1T1], with the coil1 region swapped to that of TANGO1, can still bind to TANGO1. Interestingly, the coil2 region of cTAGE5 also has a capacity to interact with Sec12 in vitro, although coimmunoprecipitation experiments fail to detect the interaction. It could be because expressed coil2 of cTAGE5 efficiently binds to the endogenous TANGO1 in a coimmunoprecipitation assay and then competes with the interaction with Sec12. In any case, lack of interaction between cTAGE5–[coil1T1] and Sec12 further supports the idea that cTAGE5 interacts with Sec12 via the coil1 region within cells.

Our findings that Sec12 clusters even in cells in which collagen secretion is compromised or in the suspension-cultured cells suggest that cTAGE5-mediated Sec12 concentration is not specifically organized for collagen secretion. In other words, Sec12 clustering seems not to be induced by collagen secretion. cTAGE5 thus might act as a static scaffold to tether Sec12 to the ER exit sites, unless Sec12 clustering via cTAGE5 might be regulated by other professional factors for collagen secretion, such as KLHL12 and Sedlin. Then, the questions arise whether the conventional COPII vesicles and the collagen-containing transport carriers or even the carriers containing both big and small cargoes can be formed from the same ER exit sites and, if so, how it is regulated. Interestingly, collagen transport through the Golgi stack have been extensively investigated, and it is widely accepted that collagen traverses the Golgi by cisternae progression–maturation (Luini, 2011). Recently, Beznoussenko et al. (2014) found that soluble cargoes traverse the Golgi faster than collagen by diffusion via intercisternal continuities, suggesting that collagens and conventional cargoes are transported by the same components through different mechanisms. These characteristics might also be applied to the ER–Golgi transport. Further work is necessary to uncover the process.

In conclusion, our study provides the first physiological insights into the functional significance of Sec12 concentration at the ER exit sites in mammalian cells. Sec12 localization has been extensively investigated in the budding yeast *P. pastoris*, in which Sec12 is also concentrated at ER exit sites in contrast to the diffused localization of Sec12 in another budding yeast *S. cerevisiae* (Soderholm et al., 2004; Okamoto et al., 2012). An interaction with Sec16 via the cytoplasmic domain of Pichia's Sec12 is reported to be responsible for its localization (Soderholm et al., 2004; Montegna et al., 2012). However, the reason why Sec12 localizes to ER exit sites in this species remains unknown. The mammalian Sec12 also has the potential to interact with Sec16 (Montegna et al., 2012); however, the mechanism of its localization is most likely different from that of *P. pastoris*. Mammalian Sec12 appears to adopt a unique mechanism—interaction with cTAGE5—to localize to ER exit sites. As cTAGE5 is evolutionarily conserved across vertebrates, it is tempting to speculate that cTAGE5 evolved to aid in the secretion of large cargo molecules, including collagens, which would require high amounts of activated Sar1 and the cTAGE5–TANGO1 complex to exit the ER. How collagen secretion is achieved by the cTAGE5-mediated concentrated Sec12 in coordination with other professional factors awaits further investigation.

Materials and methods

Antibodies

A female 6-wk-old Wistar rat (CLEA Japan, Inc.) was immunized with GST-tagged Sec12 (93–239 aa) in TiterMax Gold (TiterMax USA, Inc.). Splenocytes were fused with PAI mouse myeloma cells using Polyethylene Glycol 1500 (Roche). Hybridoma supernatants were screened by indirect ELISA with ColdTF-tagged Sec12 (93–239 aa) as the antigen. Positive hybridoma lines were subcloned, grown in serum-free medium (Nihon Pharmaceutical) supplemented with HT (Life Technologies), and purified with protein G–Sepharose (GE Healthcare). Purified rabbit polyclonal antibodies against cTAGE5 (cTAGE5 CC1 [118–227 aa]; cTAGE5 C terminus [CT; 791–804 aa]), TANGO1 (1,884–1,898 aa), and collagen VII (NC2 domain) were used as described previously (Saito et al., 2009, 2011). Other antibodies were purchased from the following companies: Sec16 (rabbit; Bethyl Laboratories, Inc.), KDEL (mouse; Enzo Life Sciences), β-actin (mouse; Sigma-Aldrich), EEA1 (mouse; BD), GM130 (mouse; BD), calnexin (mouse; BD), FLAG (mouse; Sigma-Aldrich), HA (rat; Roche), Sec31 (mouse; BD), and ERGIC-53 (mouse; Enzo Life Sciences).

Constructs

N-terminal FLAG-tagged cTAGE5-coil1 (61–300 aa), cTAGE5-coil2 (301–650 aa), cTAGE5 proline-rich domain (651–804 aa), and N-terminal HA-tagged Sec12 (1–386 aa) were cloned into pCMV5 vectors gifted from D. Russell (University of Texas Southwestern Medical Center, Dallas, TX). cTAGE5–[coil1T1] cDNA have been made by changing the coil1 region of cTAGE5 (61–300 aa) to that of TANGO1 (1,211–1,440 aa). For cTAGE5 rescue constructs (FLAG-tagged cTAGE5 and FLAG-tagged [coil1T1]), 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 amino acids were truncated so as not to be recognized by the cTAGE5 CT antibody for immunofluorescence experiments. cTAGE5 rescue constructs were cloned into a modified pCMV5 vector, in which the promoter had been truncated for mild expression level.

siRNA oligonucleotides (oligos)

Stealth select siRNAs for cTAGE5, Sec12, collagen VII, and Sec16, were purchased from Life Technologies. The oligo sequences used were cTAGE5 siRNA (1,825; HSS106514), 5′-CCGCCAGGACAAUCAUAUCCUGAUU-3′; cTAGE5 siRNA (91; HSS181098), 5′-GACCAGAUUCUAAUCUUUAUGGUUU-3′; Sec12 siRNA (1,075; HSS145462), 5′-CCAGAGCUCCUUGGGUCCCAUGAAA-3′; Sec12 siRNA (446; HSS173406), 5′-CAGACUUUAGCUCCGAUCCACUGCA-3′; collagen VII siRNA (1,933; HSS102136), 5′-UGAUGUCUGUGGCAGUAGAGUCUGG-3′; collagen VII siRNA (2,938; HSS102137), 5′-AGCUUGAGAUCCCUGGAAGUGUCUG-3′; Sec16 siRNA (2,177; HSS145369), 5′-GGGCGCAAAGUGAGCUGCCAGAUUU-3′; and Sec16 siRNA (5,393; HSS145371), 5′-CCCUGCCUAGUUUCCAGGUGUUUAA-3′. For control siRNA, Negative Universal Control Med #1 (catalog no. 12935-300; Life Technologies) was used. The number in the parentheses represents the starting base pair of the target sequence.

Immunoprecipitation and Western blotting

The experiments were essentially performed as described previously (Saito et al., 2009, 2011). In brief, extracted cells were centrifuged at 100,000 *g* for 30 min at 4°C. The cell lysates were immunoprecipitated with antibodies conjugated with protein A– or protein G–Sepharose beads (GE Healthcare). The beads were washed with TBS/0.1% Triton X-100 for five times and processed for sample preparation. All the Western blotting and immunofluorescence figures were representative of at least three individual experiments.

In vitro binding assay

MBP, MBP-tagged cTAGE5-coil1 (61–300 aa), cTAGE5-coil2 (301–650 aa), TANGO1-coil1 (1,211–1,440 aa), and TANGO1-coil2 (1,440–1,650 aa) were expressed in *Escherichia coli* and purified with amylose resin. MBP fusion proteins were conjugated to amylose resin and incubated with FLAG-Sec12 purified from Sf9 cells. Beads were washed with TBS/0.1% Triton X-100 for four times followed by elution with maltose.

Recombinant protein purification

Baculovirus encoding His_6-Sar1A, FLAG-Sec12, cTAGE5-FLAG, and His_6-TANGO1 CT region (1,211–1,907 aa) were made with Bac-to-Bac Baculovirus Expression System according to manufacturer's protocol (Life Technologies). Sf9 cells infected with virus were collected. Purification of each protein was made either by Ni Sepharose Fast Flow (GE Healthcare) for His-tagged proteins or FLAG M2 Agarose beads (Sigma-Aldrich) for FLAG-tagged proteins. In brief, cells were extracted and centrifuged at 100,000 *g* for 30 min at 4°C. Cell lysates were incubated with corresponding resins and washed. Elution was made either with imidazole for His-tagged proteins or FLAG peptide for FLAG-tagged proteins. The buffers were exchanged by a Superdex 200 HR 10/30 or PD-10 column.

GDP dissociation assay

500 nM His_6-Sar1A was incubated for 30 min at 30°C in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% Lubrol, and 5 µM [^{3}H]GDP and chilled on ice to load [^{3}H]GDP on Sar1. For GDP dissociation, His_6-Sar1A was diluted to 100 nM in the buffer consisting of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.1% Lubrol, 1 µM [^{3}H]GDP, and 200 µM GTP in the presence or absence of 15 nM Sec12, 100 nM cTAGE5, and 100 nM TANGO1 CT for the indicated times at 30°C. The reaction was stopped by adding ∼2 ml of ice–cold TMN buffer consisting of 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 20 mM $MgCl_2$ followed by filtration on nitrocellulose filters (Advantec). The filters were washed three times with TMN buffer. [^{3}H]GDP retained on the Sar1 was measured (Saito et al., 2005).

Density gradient fractionation

siRNA-treated cells were washed with buffer consisting of 0.25 M sucrose, 10 mM triethanolamine, and 1 mM EDTA twice. Cells were scraped and washed with buffer A consisting of 0.21 M sucrose, 8.5 mM triethanolamine, 1.5 mM Tris-HCl, pH 7.4, 0.75 mM KCl, 19.2 mM NaCl, and 1 mM EDTA. Cells in buffer A were homogenized with 12 passages through a 25-guage needle and centrifuged at 1,000 *g* for 5 min at 4°C. Supernatants were loaded onto the 0–26% OptiPrep (AXIS-SHIELD) density gradient and centrifuged with a rotor (MLS-50; Beckman Coulter) at 50,000 rpm for 2 h. Fractions were collected from top to bottom by a piston gradient fractionator (Biocomp Instruments).

Immunofluorescence microscopy

Immunofluorescence microscopy analysis was performed as described previously (Saito et al., 2009, 2011). For cells fixed with cold methanol, cells grown on coverslips were washed with PBS, fixed with methanol (6 min at −20°C), and then 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 room temperature) and then washed with PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature 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 room temperature) followed by incubation with Alexa Fluor–conjugated secondary antibodies (Alexa Fluor 488, Alexa Fluor 568, and/or Alexa Fluor 647 for 1 h at room temperature). Images were acquired with confocal laser scanning microscopy (Plan Apochromat 63×/1.40 NA oil immersion objective lens; LSM 700; Carl Zeiss). The acquired images were processed with Zen 2009 software (Carl Zeiss). For Figs. 3 (B and D) and S2 B, images were collected by confocal laser-scanning microscopy (HC Plan Apochromat CS2 63×/1.40 NA oil immersion objective lens; TCS SP8; Leica), equipped with detectors for photon counting (HyD; Leica). Images were processed with LAS-AF software (Leica). All imaging was performed at room temperature.

Quantification of collagen VII staining

Quantification of collagen VII accumulation was essentially performed as described previously (Saito et al., 2011). Stained cells were analyzed by epifluorescence microscopy (EC Plan Neofluar 40×/0.75 NA objective lens; Axio Imager.M1; Carl Zeiss) and processed with AxioVision software (Carl Zeiss). Area calculation and intensity scanning were performed by ImageJ software (National Institutes of Health). HSC-1 cells were treated with control or cTAGE5 siRNA. After 24 h, the FLAG-mock construct was transfected for control siRNA–treated cells. For cTAGE5 siRNA–treated cells, FLAG-tagged cTAGE5 or cTAGE5–[coil1T1] rescue constructs were transfected. After 48 h, cells were fixed and stained with collagen VII and FLAG antibodies. Typically, >90% of the cells showed efficient reduction of cTAGE5 expression by siRNA transfection. In contrast, <10% of the cells can be transfected with FLAG-tagged rescue constructs. The 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.

Metabolic labeling

Control and cTAGE5 siRNA–treated or untreated HeLa cells were cultured in DMEM without L-methionine and L-cysteine for 1 h and then pulsed with 50 µCi [^{35}S]methionine for 1 h. The cells were washed with PBS for five times and then chased with DMEM containing 10 mM L-methionine. The medium was collected at the indicated times (0, 30, 60, and 90 min) after the chase and analyzed by SDS-PAGE followed by autoradiography.

Cell culture and transfection

HeLa, A431, HSC-1, and 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum. Raji and Jurkat cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 10 mM Hepes, pH 7.4, and 1 mM sodium pyruvate. Lipofectamine RNAiMAX (Invitrogen) was used for transfecting siRNA. For plasmids transfection, Lipofectamine 2000 (Invitrogen) or FuGENE 6 (Roche) was used.

Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from siRNA-treated HSC-1 cells by RNAiso plus (Takara Bio Inc.). cDNA was prepared by ReveTra Ace qPCR RT Master Mix with genomic DNA Remover (Toyobo, Ltd.). qRT-PCR was performed with THUNDERBIRD SYBR quantitative PCR Mix (Toyobo, Ltd.) using StepOnePlus RT-PCR System (Applied Biosystems) according to the manufacturer's protocol. Data were normalized with β-actin. Primers used for RT-PCR were collagen VII, 5′-GCTGGTGCTGCCTTTCTCT-3′ and 5′-TCCAGGCCGAACTCTGTC-3′; and β-actin, 5′-CCAACCGCGAGAAGATGA-3′ and 5′-CCAGAGGCGTACAGGGATAG-3′.

Online supplemental material

Fig. S1 shows coimmunoprecipitation of cTAGE5 with different Sec12 monoclonal antibodies, immunofluorescence of Sec12 in the presence of blocking proteins, and Sec12 localization in BFA-treated cells. Fig. S2 shows Sec12 localization in TANGO1 knockdown cells and colocalization analysis of Sec12 and ERGIC-53 in cTAGE5-depleted cells. Fig. S3 shows Sec12 localization in Sec16-depleted cells. Fig. S4 shows collagen VII mRNA content in cTAGE5 knockdown cells. Fig. S5 shows GM130 staining in cTAGE5-depleted cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201312062/DC1.

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