

The tomosyn homologue, Sro7, is a direct effector of the Rab GTPase, Sec4, in post-Golgi vesicle tethering

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ABSTRACT The tomosyn/Sro7 family is thought to play an important role in cell surface trafficking both as an effector of Rab family GTPases and as a regulator of plasma-membrane SNARE function. Recent work has determined the binding site of GTP-bound Sec4 on Sro7. Here we examine the effect of mutations in Sro7 that block Sec4 binding in determining the role of this interaction in Sro7 function. Using an *in vitro* vesicle:vesicle tethering assay, we find that most of Sro7's ability to tether vesicles is blocked by mutations that disrupt binding to Sec4-GTP. Similarly, genetic analysis demonstrates that the interaction with Sec4 is important for most of Sro7's functions *in vivo*. The interaction of Sro7 with Sec4 appears to be particularly important when exocyst function is compromised. This provides strong evidence that Sro7 and the exocyst act as dual effector pathways downstream of Sec4. We also demonstrate that Sro7 tethering requires the presence of Sec4 on both opposing membranes and that homo-oligomerization of Sro7 occurs during vesicle tethering. This suggests a simple model for Sro7 function as a Rab effector in tethering post-Golgi vesicles to the plasma membrane in a pathway parallel to that of the exocyst complex.

Monitoring Editor

Thomas F. J. Martin
University of Wisconsin

Received: Feb 22, 2018

Revised: Apr 2, 2018

Accepted: Apr 10, 2018

INTRODUCTION

The Rab GTPase Sec4 is required for the normal delivery and targeting of post-Golgi vesicles to sites of polarized growth at the plasma membrane. The vesicle docking or tethering stage requires Sec4, the multisubunit complex known as the exocyst, and the tomosyn homologues Sro7/Sro77 (Bowser *et al.*, 1992; Lehman *et al.*, 1999). The exocyst complex is composed of eight subunits, Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo84, and Exo70 (Terbush *et al.*, 1996; Guo *et al.*, 1999a). The initial docking event is thought to involve either the direct interaction of vesicle-bound GTP-Sec4 with the Sec15

component of the exocyst or a parallel interaction between GTP-Sec4 and the tomosyn homologues, Sro7/Sro77 (Guo *et al.*, 1999b; Grosshans *et al.*, 2006; Watson *et al.*, 2015). In both cases, the initial docking event is thought to be linked to the SNARE-mediated fusion event by promoting the localized assembly of SNARE monomers into fusion-competent complexes at sites of polarized growth (Hattendorf *et al.*, 2007; Wu *et al.*, 2008). Structural data for Sro7 have suggested that SNARE assembly is tightly linked to the interaction of the C-terminal autoinhibitory tail of Sro7 with the N-terminal propeller (Hattendorf *et al.*, 2007). This interaction specifically competes with binding of the SNARE domain of Sec9 to the N-terminal propeller of Sro7, allowing the SNARE domain of Sec9 to engage in trans-SNARE complex formation when the autoinhibitory tail of Sro7 binds back to the N-terminal propeller (Hattendorf *et al.*, 2007).

Overexpression of either the Sec15 subunit of the exocyst or Sro7 *in vivo* results in the formation of a large cluster of post-Golgi vesicles and cell lethality (Salminen and Novick, 1989; Rossi and Brennwald, 2011). This result depends on the function of Sec4 in its GTP-bound state, but for Sro7 is independent of SNARE proteins (Rossi and Brennwald, 2011; Rossi *et al.*, 2015). We reconstituted the vesicle:vesicle tethering process for Sro7 *in vitro* using purified Sro7 protein and isolated post-Golgi vesicles and showed that the *in vitro* process closely mirrors the requirements *in vivo* (Rossi *et al.*, 2015). Recent work from the laboratory has identified a binding site

This article was published online ahead of print in MBoC in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E18-02-0138>) on April 18, 2018.

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Abbreviations used: DSP, dithiobis(succinimidyl propionate); GDP, guanosine 5'-diphosphate; GST, glutathione S-transferase; GTPγS, guanosine 5'-3-O-(thio)triphosphate; GTPase, guanosine 5'-triphosphatase; SNARE, soluble N-ethylmaleimide-sensitive factor adaptor protein receptor; t-SNARE, target membrane SNARE; YPD, yeast peptone dextrose.

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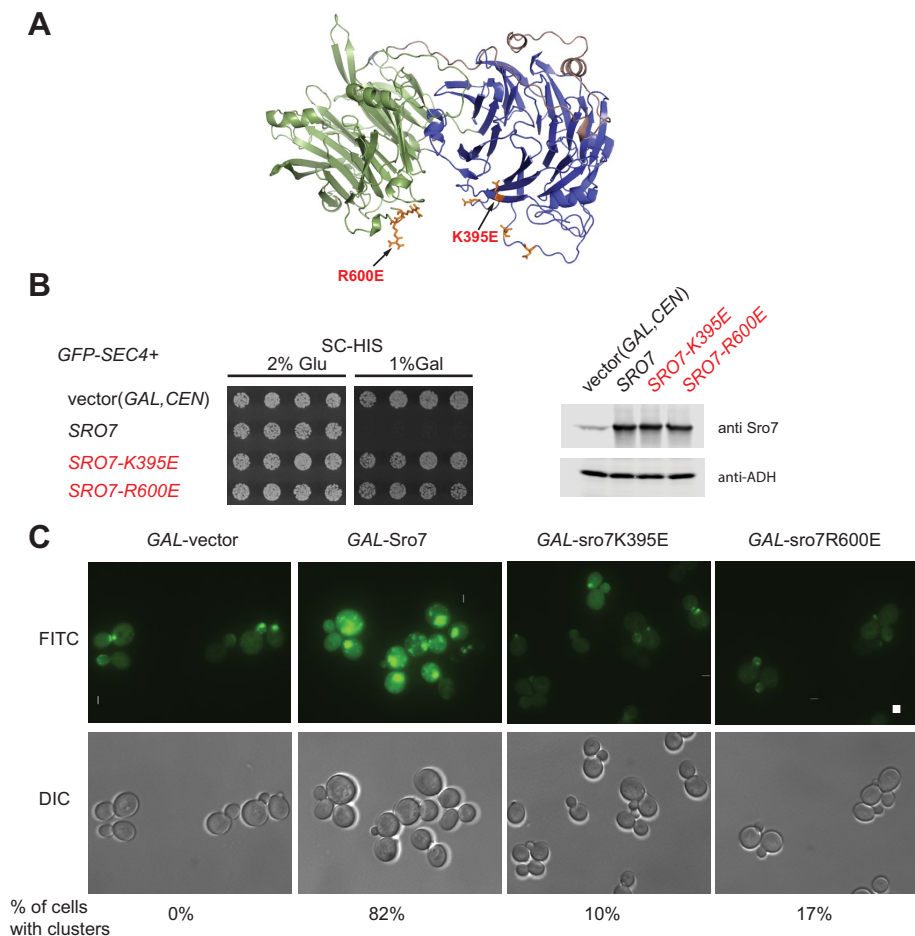


FIGURE 1: Sro7 mutants that fail to bind Sec4-GTP in vitro fail to induce post-Golgi vesicle clustering and cell lethality when overexpressed in vivo. (A) A ribbon diagram of Sro7 highlighting two (shown in red) of the six mutations (shown in orange) that affect the interaction between Sro7 and Sec4-GTP. The N-terminal β -propeller is shown in blue and the C-terminal β -propeller is green. (B) A wild-type yeast strain expressing GFP-Sec4 (CEN) was transformed with vector only or plasmids expressing wild-type Sro7, Sro7-K395E, and Sro7-R600E from a GAL promoter (GAL, CEN). Four individual colonies were picked and transferred to selective media in the presence of glucose or galactose. Western blot analysis of glass bead lysates generated from equal absorbance units of the same strains induced for 6 h in galactose is shown to the right. (C) Wild-type yeast cells expressing GFP-Sec4(CEN) and plasmids (CEN) expressing Sro7, Sro7-K395E, Sro7-R600E, or vector only from a GAL promoter were grown overnight in selective media with 3% raffinose and induced for 6 h in 1% galactose before being analyzed by fluorescence microscopy. Scale bar, 2 μ m. More than 40 small budded cells were scored for the presence of a bright patch of florescence in the mother cell; the resulting percentages are shown under the DIC images in C.

for Sec4-GTP on the surface of Sro7 (Watson *et al.*, 2015). This site resides in a cleft between the two β propellers that form the core structure of the conserved family of lethal giant larvae/tomosyn proteins. Charge-reversal mutations in this region of Sro7 resulted in mutant Sro7 proteins that failed to bind GST-Sec4-GTP in vitro and function in suppression studies of *sec15-1* mutant cells in vivo (Watson *et al.*, 2015).

To understand the role of Sro7 as an effector of Sec4 function, we make use of several structure-based mutants in Sro7, which we have demonstrated are specifically defective in binding to Sec4-GTP. Using these mutants, we demonstrate that Sro7 binding to Sec4 is specifically required for the ability of Sro7 to fully function in mediating vesicle tethering in vivo and in vitro. Additionally, we show that this interaction is also important in vivo when the cell harbors muta-

tions in a number of subunits of the exocyst complex—providing further evidence for a model where Sro7 and the exocyst act as dual effector pathways downstream of Sec4. Using the in vitro vesicle:vesicle tethering assay, we also demonstrate that Sro7 tethering requires the presence of Sec4 on both opposing membranes, and we present evidence that homo-oligomerization of Sro7 occurs during the process of vesicle tethering. This suggests a simple model for Sro7 function as a Rab effector in tethering post-Golgi vesicles in a pathway that is likely to function in parallel to that of the exocyst complex.

RESULTS

Sro7 mutants that fail to bind Sec4-GTP in vitro fail to induce post-Golgi vesicle clustering and cell lethality when overexpressed in vivo

We have recently made use of a structure-based approach to identify the surface contact sites on Sro7 responsible for its interaction with the GTP-bound form of the Rab GTPase Sec4 (Watson *et al.*, 2015). The surface mutagenesis was followed by in silico docking studies and additional surface mutagenesis of both Sro7 and Sec4 to identify and validate a precise docking model for the interaction of Sec4-GTP and its effector Sro7. This model, shown in Figure 1A, shows that Sec4-GTP binds within a cavity or “cleft” formed by the intersection of the two β propeller structures of Sro7 (Watson *et al.*, 2015). Two charge-reversal mutants in Sro7 (Sro7-K395E and Sro7-R600E)—one on each side of this cleft—both individually and combined block Sro7’s interaction with Sec4-GTP in vitro (Watson *et al.*, 2015; Figure 2A) but had surprisingly little effect on their function as the sole source of Sro7/Sro77 in vivo (Watson *et al.*, 2015). We speculated that this could be due to the functional redundancy between Sro7 and the exocyst tethering complex, which are both thought to promote Sec4-dependent post-Golgi vesicle tethering to the plasma membrane

(Grosshans *et al.*, 2006; Rossi *et al.*, 2015; Watson *et al.*, 2015). Alternatively, this finding could reflect the fact that Sro7 binding to Sec4 does not represent a critical aspect of its function in the cell. We therefore set out to examine the effects of Sec4-binding mutants on the ability of Sro7 to function, using an extensive set of in vivo and in vitro assays (Rossi *et al.*, 2015; Rossi and Brennwald, 2011).

One simple in vivo assay for Sro7 function in vesicle tethering comes from our previous observation that acute *GAL1/10* overexpression of Sro7 results in a dramatic accumulation of large clusters of post-Golgi vesicles within the cell, which due to their size are detectable by both electron and fluorescence microscopy (Rossi and Brennwald, 2011). The post-Golgi vesicle clustering phenotype is so severe that it also results in the inability of cells to grow in the presence of galactose. We have shown that vesicle clustering depends

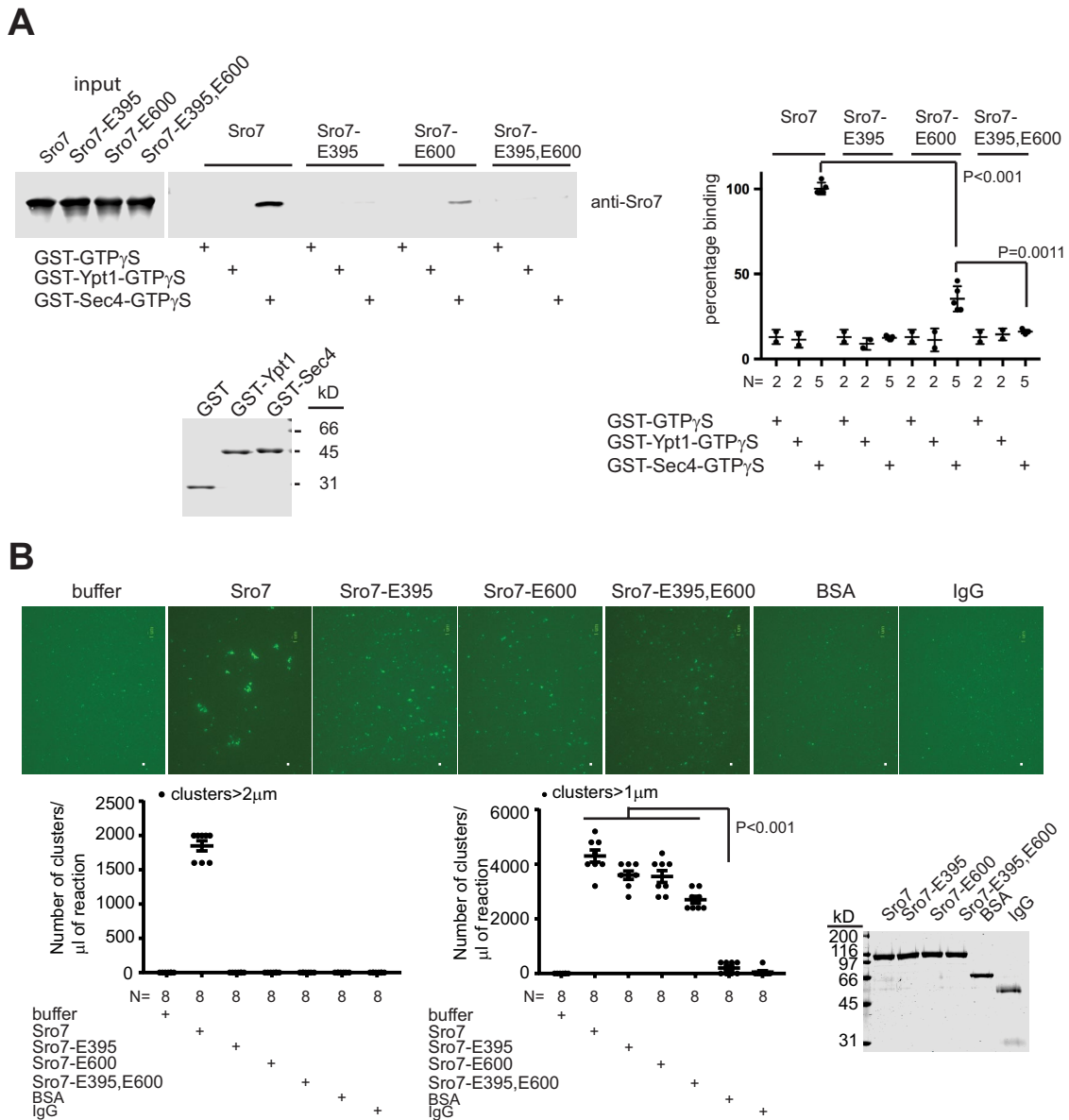


FIGURE 2: Biochemical characterization of Sro7-E395, Sro7-E600, and Sro7-E395,E600 mutant proteins shows that loss of Sro7 binding to Sec4-GTP correlates with a marked defect in vesicle:vesicle tethering in vitro. (A) Wild-type Sro7, Sro7-K395E, Sro7-R600E, and Sro7-K395E;R600E proteins were purified and compared at identical concentrations (1 μ M) in a pull-down assay with GST-Sec4-GTP using GST-GTP and GST-Ypt1-GTP as controls. The Coomassie stain of the GST fusion proteins used and the quantitation of the binding showing SD from two independent experiments are also shown. Percent binding was expressed using wild-type Sro7 binding at 100%. *p* values were obtained using a two-tailed Student's *t* test. (B) Purified wild-type Sro7 and Sro7-K395E, Sro7-R600E and the double mutant Sro7-K395E;R600E proteins were analyzed in a vesicle:vesicle tethering assay using the mutant *sec6-4* strain expressing GFP-Sec4 as a source of vesicles. Scale bar, 1 μ m. Identical concentrations of IgG and BSA were used as controls. Quantitation for the vesicle:vesicle tethering assay was expressed as numbers of vesicle clusters of two different size groups/ μ l for each tethering reaction. Error bar represents SD obtained from quantitation of images taken at 60 \times magnification. *p* values were obtained using a two-tailed Student's *t* test. A Coomassie stain shows purified proteins used in the vesicle:vesicle tethering assay in B.

on the presence of functional Sec4-GTP in the cell, as it does not occur in a *sec4-8* mutant or a *sec2-41* mutant that encodes the sole guanine nucleotide exchange factor (GEF) for Sec4 in the cell (Walch-Solimena *et al.*, 1997; Rossi and Brennwald, 2011). To examine the effect of the Sec4-binding mutants on the ability of Sro7 to cluster vesicles in this assay, we introduced two alleles of SRO7 (*sro7-K395E* and *sro7-R600E*) into the cell behind the *GAL1/10* promoter and examined the pattern of Sec4 fluorescence (a marker of post-

Golgi vesicles) and cell growth following induction on galactose containing media. As shown in Figure 1B, while wild-type SRO7 blocked growth on YP-Gal media, both of the single Sro7 charge-reversal mutants *sro7-K395E* and *sro7-R600E* demonstrated normal growth on YP-Gal plates. Western blot analysis on lysates from the induced strains confirmed that all the SRO7 alleles were expressed at comparable levels following growth in galactose containing media (Figure 1B). We also determined the effect of overexpression of

the mutant alleles of Sro7 on post-Golgi vesicle clustering by fluorescence microscopy. As previously observed (Rossi and Brennwald, 2011), wild-type Sro7 causes post-Golgi vesicle clustering, which appears as a bright patch of GFP-Sec4 fluorescence in the mother cell (Figure 1C). However, similarly to vector only, both the *sro7-K395E* and *sro7-R600E* mutants failed to form bright patches of GFP-Sec4 in the cell when overexpressed from the *GAL1/10* promoter, and Sec4-GFP localized to sites of polarized growth in these cells (Figure 1C). Quantitation of these results, shown in Figure 1C, demonstrated that mutants in Sro7 that fail to bind Sec4-GTP *in vitro* also fail to induce vesicle clustering *in vivo*. This strengthens the notion that post-Golgi vesicle:vesicle tethering *in vivo* requires physical interaction between GTP-bound Sec4 and its downstream effector Sro7.

Sro7 mutants that cannot bind Sec4-GTP fail to efficiently tether post-Golgi vesicles *in vitro*

To examine the biochemical effect of Sro7 binding to the Sec4 GTPase on its role in vesicle tethering, we made use of an *in vitro* system that we developed, which reconstitutes Sro7-mediated post-Golgi vesicle:vesicle tethering by utilizing purified Sro7 protein and vesicles obtained from well-characterized secretory mutants that are specifically labeled with fluorescent probes (Rossi *et al.*, 2015). The vesicle:vesicle tethering activity, which we observe by fluorescence microscopy, is dependent on the addition of purified Sro7 protein, MgCl₂, and GTPγS. Importantly, we have shown that vesicle:vesicle tethering requires functional Sec4-GTP on vesicle membranes but is independent of either v-SNARE Snc1/2 or t-SNARE Sso1/2 proteins (Rossi *et al.*, 2015). To compare the activity of wild-type Sro7 with that of the Sro7 mutants that fail to bind Sec4-GTP *in vitro* in the vesicle:vesicle tethering assay, we purified wild-type Sro7, two of the original mutant proteins, Sro7-K395E and Sro7-R600E, and the double mutant, Sro7-K395E;R600E, using a protein A tag purification method (Rossi *et al.*, 2015; Watson *et al.*, 2015). We first examined the ability of each of the purified proteins to specifically bind Sec4-GTP. Consistent with our previous analyses, all three proteins show a dramatic decrease in their ability to bind Sec4-GTP with only Sro7-R600E demonstrating any detectable binding to Sec4 (Figure 2A). We next analyzed the activity of the purified proteins in the vesicle:vesicle tethering assay. As previously shown, addition of wild-type Sro7 protein to the vesicles resulted in the formation of large (>2 μm) fluorescently labeled puncta, which were visualized by fluorescein isothiocyanate (FITC) (GFP-Sec4) filter sets (Figure 2B). In contrast, the addition of identical amounts of Sro7-K395E and Sro7-R600E or the double mutant Sro7-K395E;R600E was unable to promote efficient vesicle:vesicle tethering—seen as puncta >2 μm. However, we were able to detect a less efficient vesicle:vesicle tethering activity with the mutant proteins, which was seen as the appearance of small puncta between 1 and 2 μm in size, which were absent when BSA or immunoglobulin G (IgG) was used in the assay in place of Sro7 (Figure 2B). Taken together, the biochemical data confirm that direct binding of Sro7 to Sec4-GTP is important for efficient *in vitro* vesicle tethering but that—surprisingly—some vesicle:vesicle tethering activity is retained in these mutants despite the fact that all detectable Sec4-binding activity is lost (Watson *et al.*, 2015).

The residual tethering seen with Sro7-K395E, Sro7-R600E, and Sro7-K395E;R600E mutant proteins is inhibited by the addition of t-SNARE Sec9 to the assay

Previous work from our laboratory has demonstrated that the ability of purified Sro7 to induce vesicle:vesicle tethering *in vitro* depends on both the presence of Sec4 on vesicles and the conformational state of the Sro7 protein used in the assay. Mutant forms of Sro7 that

fail to adopt a closed conformation in which the C-terminal tail binds back to the N-terminal propeller fail to induce vesicle tethering *in vitro* and *in vivo* (Rossi *et al.*, 2015). Vesicle:vesicle tethering is also inhibited if Sro7 is preincubated with purified Sec9 as the t-SNARE. Sec9 binds to the Sro7 N-terminal propeller and shifts Sro7 to adopt an open conformation in which the C-terminal tail cannot engage the N-terminal propeller (Rossi *et al.*, 2015). To test whether the residual clustering seen with the Sro7 mutants was also subject to inhibition with the t-SNARE Sec9, we preincubated Sec9 with both wild-type Sro7 and the Sro7 mutants before performing the vesicle:vesicle tethering assay. Figure 3A demonstrates that any residual vesicle tethering seen with Sro7-K395E, Sro7-R600E, and Sro7-K395E;R600E is inhibited by Sec9, suggesting that the residual tethering seen in the vesicle:vesicle tethering assay is due to low levels of bona fide Sro7 tethering activity, as it is subject to the same inhibitory effect of Sec9 as demonstrated by the wild-type Sro7 protein.

SEC4 suppression of the exocyst mutant *sec15-1* requires physical interaction between Sec4 and Sro7

One of the earliest and strongest genetic relationships among the late secretory genes was the observation that a single extra copy of *SEC4* is able to potentially rescue a temperature-sensitive defect in the Sec15 subunit of the exocyst complex (Salminen and Novick, 1989). Duplication of *SEC4* was sufficient to completely rescue the temperature sensitivity associated with the *sec15-1* mutant strain. Our findings here, along with previous genetic data (Lehman *et al.*, 1999; Grosshans *et al.*, 2006), strongly support a model in which Sro7 functions in Sec4-dependent vesicle tethering in a pathway that acts in parallel to that of the exocyst complex. This suggests a simple model for the potent effect of *SEC4* duplication on the *sec15-1* and other exocyst mutants observed almost 30 years ago (Salminen and Novick, 1989). In this model (Figure 4B), an extra copy of *SEC4* leads to up-regulation of a parallel effector pathway mediated by Sro7, which therefore reduces the dependence on the exocyst complex for overall Sec4 effector function. To test this model directly, we wanted to determine whether the Sro7 effector pathway was required for this suppression, and in particular if mutations that effect the interaction of Sec4 with Sro7 effect the suppression of *sec15-1* by *SEC4* duplication. We generated a *sec15-1; sro7Δ; sro77Δ* plasmid shuffle strain containing a single copy of wild-type *SRO7* on a *CEN*, *URA3* plasmid (this was a necessary step, as the triple mutant strain is nonviable). This strain was then transformed with wild-type *SRO7*, *SRO7-K395E*, or *SRO7-R600E* on a *CEN*, *HIS3* plasmid and the *URA3* plasmid containing wild-type *SRO7* was evicted on 5-fluoroorotic acid (5-FOA) plates. The loss of the *URA3*-containing plasmid on 5-FOA plates allowed the generation of four distinct *ura3* minus strains, which we then transformed with a *SEC4*, *CEN*, *URA3* plasmid (resulting in *SEC4* duplication) to allow us to score for *sec15-1* suppression. The results, shown in Figure 4A, demonstrate that while the presence of wild-type *SRO7* supports strong suppression of *sec15-1* by *SEC4* duplication, both *sro7-K395E* and *sro7-R600E* fail to support suppression of the *sec15-1* mutation by *SEC4* duplication. This demonstrates not only that *SEC4* suppression acts through the Sro7 effector pathway, but also that the physical interaction between Sec4-GTP and Sro7 is important for the function of this parallel effector pathway (Figure 4B).

Effects of *sro7-K395E*, *sro7-R600E*, and *sro7-K395E;R600E* mutations on multicopy suppression of late-acting Rho GTPase and secretory mutants

SRO7 was initially identified as a high-copy suppressor of loss of rho3 function and also demonstrated strong suppression of a

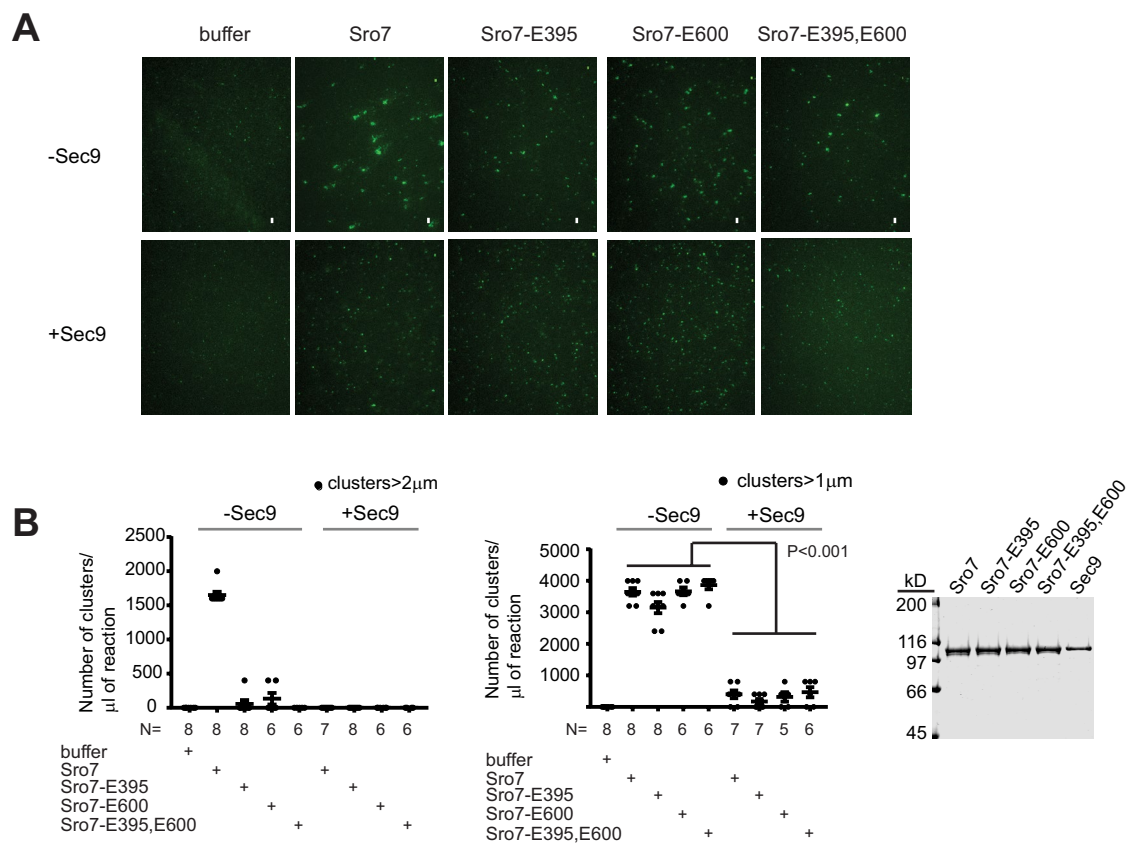


FIGURE 3: Residual vesicle:vesicle tethering mediated by Sro7K395E, Sro7R600E, and Sro7K395E,R600E mutant proteins is inhibited by purified Sec9. (A) Wild-type Sro7, Sro7-K395E, Sro7-R600E, and Sro7-K395E;R600E proteins were purified and compared at identical concentrations (1 μ M) in the vesicle:vesicle tethering assay using vesicles expressing GFP-Sec4 obtained from a *sec6-4* mutant strain. Vesicle:vesicle tethering assays were conducted in the presence of Sec9 (1 μ M) or buffer only. Scale bar, 1 μ m. (B) Quantitation for the vesicle:vesicle tethering assay was expressed as numbers of vesicle clusters of two different size groups/ μ l each tethering reaction. Error bar represents SD from quantitation of images taken at 60 \times magnification. *p* values were obtained using a two-tailed Student's *t* test. A Coomassie stain shows purified proteins used in the vesicle:vesicle tethering assay in A.

number of late secretory mutants, as well as a secretory-deficient allele of *CDC42*, *cdc42-6* (Kagami *et al.*, 1997; Lehman *et al.*, 1999; Adamo *et al.*, 2001; Wu *et al.*, 2010). We therefore wanted to make use of the Sec4 binding-site mutants in Sro7 to determine if the ability of Sro7 to suppress the growth defects of these strains is tightly linked to its ability to bind to Sec4. All three Sec4-binding mutants were introduced onto high-copy (2 μ) plasmids into eight different conditionally defective yeast strains. Six of these mutants are temperature-sensitive alleles of subunits of the exocyst complex, and two of the mutants are conditional alleles in the Rho GTPases, Rho3 and Cdc42. Interestingly, while five out of the six exocyst mutants and the *cdc42-6* mutant, demonstrated that *SRO7* suppression was clearly sensitive to loss of Sec4 binding, two mutants, *exo70-113* and *rho3 Δ* , demonstrated no apparent effect on growth when either single or double mutants in the Sec4 binding sites were present in *SRO7* (Figure 5). Taken together, these genetic data suggest that while much of the ability of Sro7 to function in exocytosis is intimately tied to its role as a Sec4 effector, Sro7 likely has certain aspects of its function that do not require direct engagement with Sec4.

Sro7-mediated post-Golgi vesicle:vesicle tethering is symmetric in its Rab GTPase requirement, as the presence of Sec4 is required on each opposing membrane

To determine whether Sro7-mediated vesicle tethering is symmetric in its requirement for Sec4 on opposing membranes, we developed a version of the tethering assay using two populations of vesicles with distinct fluorescent labels. One population of vesicles contained functional GFP-Sec4 (green) derived from a *sec6-4* strain, while the other population of vesicles was generated from a *sec4-8* strain and labeled with the FM4-64 dye (red) during the vesicle isolation procedure. As a positive control, we also isolated FM4-64-labeled vesicles from a *sec6-4* strain, which results in (red) post-Golgi vesicles with wild-type Sec4 on their surface (Goud *et al.*, 1988). If Sec4 is required on only one of the two membranes in the tethering reaction, we would predict that the FM4-64-labeled vesicles obtained from the *sec4-8* mutant strain would be able to tether or "co-cluster" with the GFP-Sec4-labeled vesicles when mixed together in the tethering assay. This would be seen as dual labeled clusters forming in the presence of Sro7. In contrast, if Sec4 is symmetrically required on each vesicle surface to form a tether with another vesicle, then we would expect to see little or no detectable

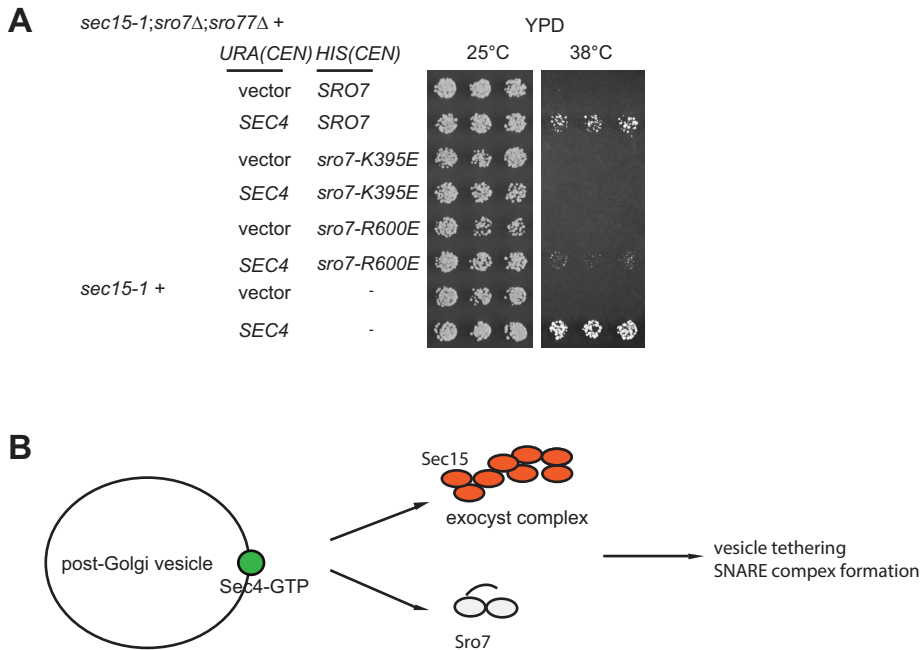


FIGURE 4: *Sec4* suppression of the *sec15-1* mutant strain requires its direct interaction with *Sro7*. (A) A triple mutant strain *sec15-1; sro7Δ; sro77Δ* expressing *SRO7*, *SRO7-K395E*, or *SRO7-R600E* (*CEN, HIS3*) was transformed with *SEC4* or vector only (*CEN, URA3*). Three individual transformants were picked and transferred onto selective media at the permissive and restrictive temperatures. The *sec15-1* mutant strain was transformed with a plasmid expressing *SEC4* or vector only (*CEN, URA3*). Three individual colonies were picked and transferred onto selective media at the permissive and restrictive temperatures. (B) A model for *Sro7* and exocyst function downstream of *Sec4*.

FM4-64 fluorescence in the GFP-*Sec4*-labeled clusters when *sec4-8* vesicles labeled with FM4-64 are used. However, one would predict that we would readily detect coincident fluorescence if FM4-64-labeled *sec6-4* vesicles (containing *Sec4*) are included in the mixing instead of FM4-64 labeled *sec4-8* vesicles (Figure 6A). In fact, the latter possibility is exactly what we find, as can be seen in Figure 6B. While *sec6-4* vesicles (with wild-type *Sec4* on their surface) readily cotether with GFP-*Sec4* vesicles, *sec4-8* vesicles not only fail to tether/cluster, but appear to interfere with GFP-*Sec4* vesicles tethering to themselves. This experiment demonstrates that *Sec4* is required on the surface of both membranes for *Sro7*-mediated tethering to occur and that the Rab requirement for *Sro7* is symmetric in the mode of tethering promoted by this effector.

Sro7*-mediated post-Golgi vesicle:vesicle tethering is accompanied by homo-oligomerization of *Sro7

The symmetry of the *Sec4* requirement in *Sro7*-mediated tethering has important mechanistic implications when this observation is combined with other recent work from our lab. Using a comprehensive surface mutagenesis and in silico docking analysis, we identified a single binding site on the surface of *Sro7* as responsible for the GTP-dependent interaction with *Sec4* (Watson *et al.*, 2015). However, the purified *Sro7* used in these assays is monomeric when examined by gel filtration or velocity sedimentation analyses (Hattendorf *et al.*, 2007). Taken together, these observations suggest that for *Sro7* to promote interactions between opposing membranes containing *Sec4*-GTP, *Sro7* is likely to form homo-oligomeric interactions (Figure 7D). To rigorously examine whether such homo-oligomeric interactions form during *Sro7*- and Rab-dependent vesicle tethering, we developed a homo-oligomerization assay using purified epitope-

tagged forms of *Sro7*. We inserted single HA or Myc epitope tags into a nonconserved region within the flexible N-terminal domain of *Sro7* (see *Materials and Methods*). We demonstrated that the epitope-tagged forms of *Sro7* were functional both by complementation of the *sro7Δ/sro77Δ* double deletion strain and by suppression of the temperature sensitivity of a *sec15-1* mutant strain. We then purified the tagged proteins as previously described (Rossi *et al.*, 2015; Figure 7C) and found, as expected, that they showed normal activity in our in vitro vesicle:vesicle tethering assay. We then used either single-tagged or mixtures of the two epitope-tagged *Sro7* proteins in the in vitro vesicle:vesicle tethering assay, followed by treatment with the cleavable cross-linking agent dithiobis (succinimidyl propionate) (DSP) and immunoprecipitations with 9E10 mAb (anti-myc) under conditions where only covalent associations between DSP-cross-linked *Sro7* molecules would be observed. The immunoprecipitations were then immunoblotted with 12CA5 mAb (anti-HA) to determine the extent of coassociation of the two tagged forms of *Sro7*. As can be seen in Figure 7A, some degree of baseline homo-oligomerization of *Sro7* occurs when the two tagged proteins are mixed in the presence of *sec6-4* or *sec4-8* vesicles kept on ice (time 0) prior to cross-linking treatment. However,

when *sec6-4* vesicles were then incubated for 45 min at 25°C in the presence of a mixture of HA and Myc-tagged *Sro7*—conditions under which robust vesicle:vesicle tethering occurs—we see a dramatic increase in the level of oligomerization between the tagged proteins (Figure 7, A and B). In contrast, when the oligomerization assay was performed with *sec4-8* vesicles and incubated for 45 min at 25°C—conditions under which no vesicle:vesicle tethering is observed—we failed to see any increase in oligomerization of the tagged proteins. This gives us clear evidence that *Sro7* homo-oligomerization is an important feature of how this protein functions during vesicle tethering. This suggests a simple model in which, following binding of monomeric *Sro7* to vesicle-associated *Sec4*-GTP, homo-oligomeric interactions between *Sro7* proteins (also bound to *Sec4*-GTP) on opposing membrane surfaces would help stabilize the tethering system (Figure 7D).

DISCUSSION

This work synthesizes and builds on two recent studies from our group: one study describing a validated structural model for the binding of the *Sec4* GTPase to the surface of *Sro7* (Watson *et al.*, 2015) and the other describing a novel in vitro assay for *Sro7* and *Sec4*-dependent vesicle:vesicle tethering (Rossi *et al.*, 2015). Here we make use of specific mutations to residues identified in this study (Watson *et al.*, 2015) that reside on each side of a cleft in *Sro7* formed by the junction of its two β -propeller structures. This cleft forms the sole binding site for *Sec4*-GTP on *Sro7*, and mutations of any of the predicted sites of contact on *Sro7* or *Sec4* result in severe loss of *Sec4* binding to *Sro7* (Watson *et al.*, 2015). As expected, these mutations block detectable *Sec4* binding in vitro, as well as most—but not all—vesicle-tethering activity in vitro. This represents the strongest

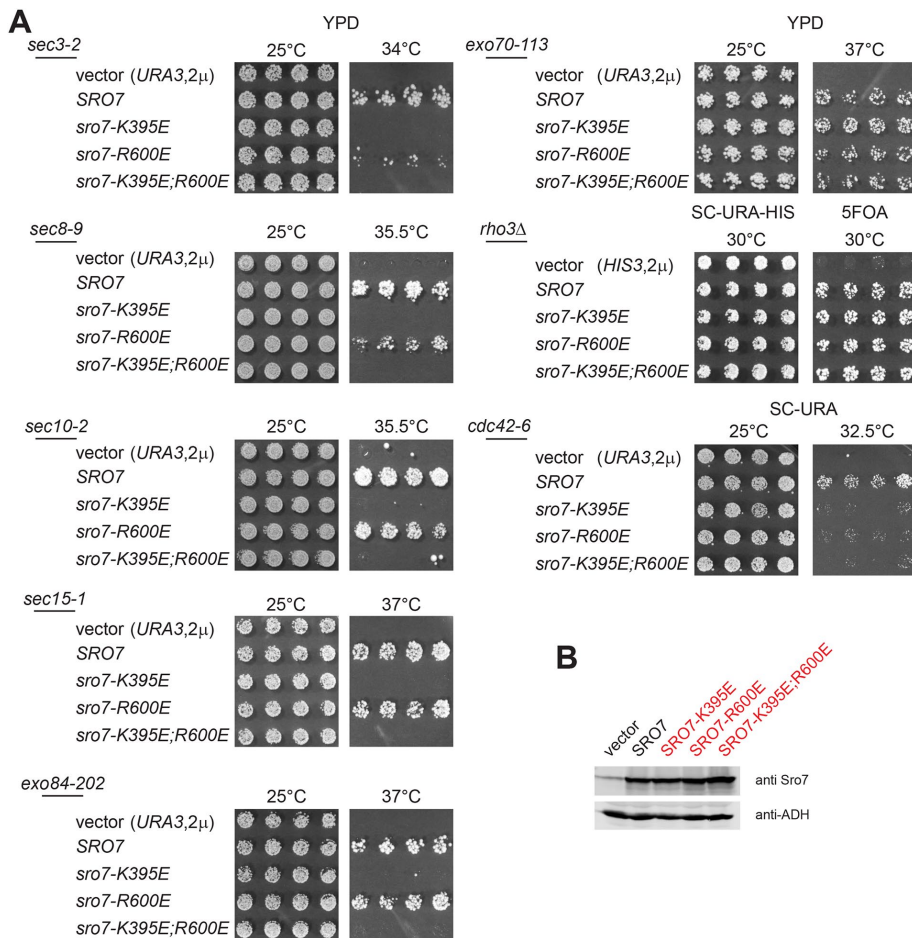


FIGURE 5: Sro7 mutants that fail to bind Sec4 can no longer suppress *cdc42-6* and a number of secretory mutants in the exocyst complex. (A) Yeast secretory mutant strains (*cdc42-6*, *sec3-2*, *sec8-9*, *sec10-2*, *sec15-1*, *exo70-113*, and *exo84-202*) were transformed with vector only (2μ, *URA3*) or plasmids expressing wild-type Sro7, Sro7-K395E, Sro7-R600E, and Sro7-K395E;R600E (2μ, *URA3*). Four individual colonies were picked and transferred to selective media at the indicated permissive and restrictive temperatures. In the case of *rho3Δ*, a plasmid shuffle strain containing Rho3 (*CEN,URA3*) was transformed with vector only (2μ, *HIS3*) or plasmids expressing wild-type Sro7, Sro7-K395E, Sro7-R600E, and Sro7-K395E;R600E (2μ, *HIS3*). The *URA3* plasmid containing wild-type Rho3 was then evicted on 5FOA plates. (B) Western blot analysis of glass bead lysates generated from equal absorbance units of a *sec8-9* strain transformed with high-copy plasmids expressing Sro7, Sro7-K395E, Sro7-R600E, and Sro7-K395E;R600E or vector only.

evidence to date that Sro7 is a direct effector of Sec4 and that direct binding of Sro7 to Sec4-GTP is required for Sro7 to mediate its vesicle-tethering activity. It is also important to note that since the exocyst complex does not copurify with the post-Golgi vesicles used in this assay, the tethering activity observed in this assay is directly mediated by Sro7, rather than indirectly through Sro7 interaction with the exocyst complex (Zhang *et al.*, 2005). Interestingly, the Sec4 binding cleft on Sro7 appears to have been conserved in mammalian tomosyn (Watson *et al.*, 2015), which was recently shown to specifically bind to Rab3A in its GTP bound form (Cazares *et al.*, 2016).

We previously made the unexpected finding that the Sro7 mutations used here had no deleterious effect on the mutant protein's ability to function as the sole source of Sro7/Sro77 in the cell (Watson *et al.*, 2015). In contrast, in the present study, we find clear evidence for loss of Sro7 function in these same Sro7 mutants by several genetic tests. Importantly, we found that when present as the sole

source of Sro7/Sro77, the Sec4-binding mutants have a profound effect on the ability of duplicated *SEC4* to suppress *sec15-1*, an essential component of the exocyst complex. The critical difference in these two results is the functionality of the exocyst effector pathway. When defects exist in the exocyst effector pathway, the Sec4 function is extremely sensitive to its ability to bind Sro7. Similarly, the sensitivity of *SRO7* dosage suppression (at least in six of the eight strains tested) to Sec4-binding mutations reinforces the importance of the direct interaction of Sec4 and Sro7 *in vivo*. Taken together, these observations significantly strengthen the genetic and biochemical evidence that Sro7 and the exocyst complex represent two direct but parallel effector pathways for Sec4 function in post-Golgi vesicle tethering.

While the data presented here and previously (Watson *et al.*, 2015) clearly demonstrate that Sec4 binding is important to Sro7's role as an effector for the Sec4 GTPase, we also find multiple pieces of genetic and biochemical data suggesting that Sro7 may also possess significant functionality in the absence of full Sec4 engagement. The genetic evidence presented here includes equally strong dosage suppression of *rho3Δ* and *exo70-113* strains by the Sec4-binding defective alleles of *SRO7*. Biochemically, we also observed a low but significant level of vesicle tethering with Sro7 mutants that are unable to bind Sec4, which was still inhibited by the addition of the t-SNARE Sec9—demonstrating that it is sensitive to the same conformational regulation that Sec4-dependent tethering reaction is. Because Sro7 is able to engage the exocyst complex through interaction with the Exo84 component, it is possible that the genetic data may involve Sro7 "piggybacking" on the ability of the exocyst complex to engage Sec4-GTP. However, because the exocyst is absent from our *in vitro* tethering assay,

it is unlikely to explain the residual tethering observed with the Sro7 mutant proteins. Instead, this activity may represent the interaction of Sro7 with other components of the vesicle, including either proteins or phospholipid components. Further studies with synthetic liposomes will be necessary to distinguish between these possibilities.

One of the important aspects of this work was further development of the *in vitro* tethering assay to allow us to examine the symmetry/asymmetry of the requirements on each membrane being tethered—in particular, the requirement for the Rab GTPase Sec4. Sec4 is known to reside normally on both secretory post-Golgi vesicles, as well as the plasma membrane (Goud *et al.*, 1988). Therefore, the normal *in vivo* tethering process mediated by Sro7 could involve either symmetric requirements for Sec4 on both vesicle and plasma membrane or require Sec4 solely on the vesicle side of the tethering reaction, with other Rab-independent interactions with

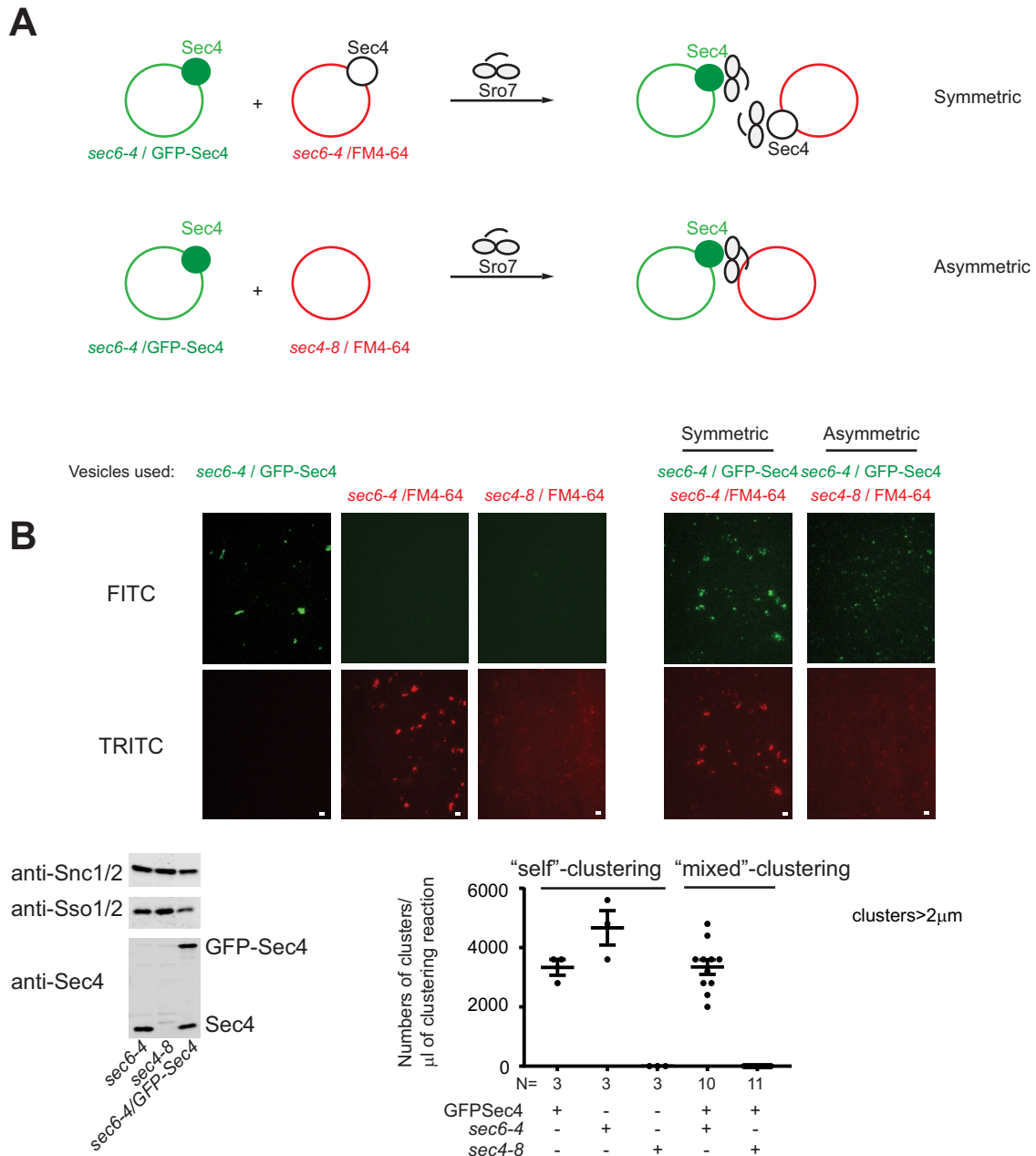


FIGURE 6: Sro7 requires the presence of Sec4 on opposing membranes to mediate vesicle:vesicle tethering in vitro. (A) Schematic showing the use of differential vesicle labelling for testing the symmetric/asymmetric requirement for Sec4 in Sro7-mediated vesicle:vesicle tethering. (B) Vesicles obtained from *sec6-4* and *sec4-8* mutant strains were labeled with the lipid dye FM4-64 and used on their own or mixed with vesicles obtained from a *sec6-4* mutant strain expressing GFP-Sec4 in an in vitro vesicle:vesicle tethering assay with purified Sro7 (2 μM), MgCl₂ (3 mM), and GTPγS (1 mM). Scale bar, 2 μm. Total vesicle material used for the vesicle:vesicle tethering assay was monitored by Western blot analysis with vesicle marker proteins (Sec4, Snc1/2, and Sso1/2). Quantitation of the assay is expressed as the number of vesicle clusters above 2 μm/μl for each tethering reaction. “Self”-clustering refers to clusters that are observed in either FITC or TRITC channels. “Mixed”-clustering refers to vesicle clusters that can be observed in both FITC and TRITC channels. Error bar represents the SD obtained from quantitation of images taken at 60× magnification.

the plasma membrane—similar to the proposed mechanism for exocyst-dependent tethering (Wu *et al.*, 2008; Wu and Guo, 2015; Heider and Munson, 2012). Importantly, the results of our assays demonstrated a clear requirement for Sec4 on both membranes involved in tethering. Because our structural examination of Sec4 binding on Sro7 revealed the presence of only a single binding site for Sec4 (Watson *et al.*, 2015), this immediately suggested to us that

oligomerization of Sro7 would likely play a role in the tethering process. This led us to develop a method for monitoring oligomerization of Sro7 utilizing epitope-tagged forms of Sro7 and a cleavable cross-linking agent that we incorporated into the in vitro tethering assay. This allowed us to rigorously determine the extent of homooligomeric association both prior and during the in vitro tethering process. Interestingly, we observed significant oligomerization

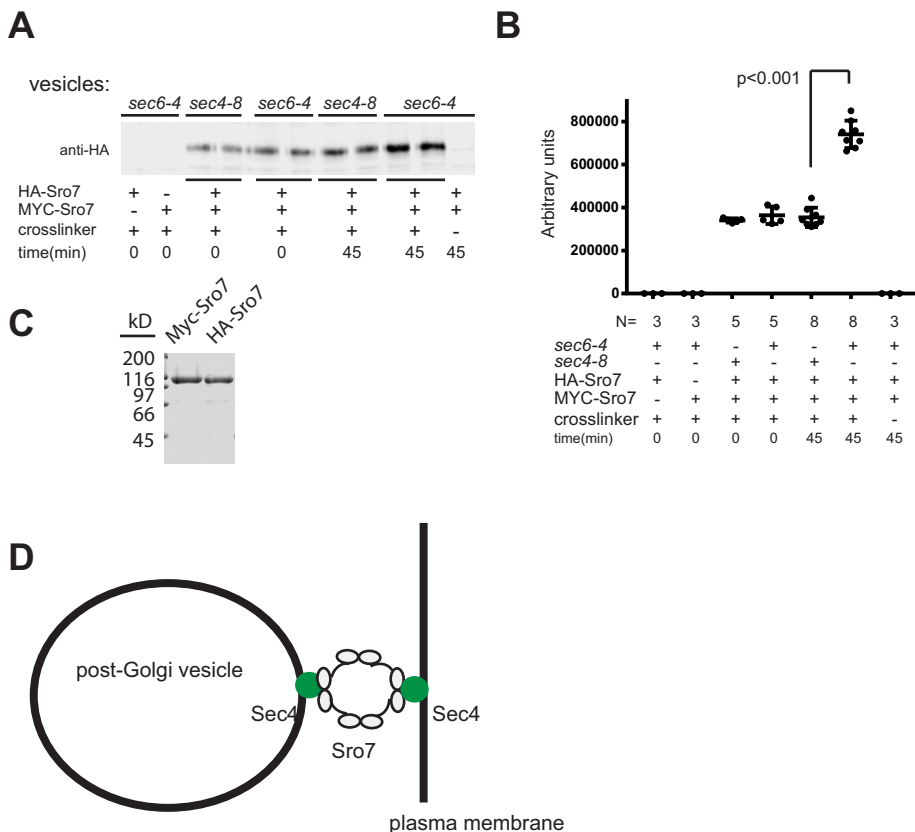


FIGURE 7: Sro7-mediated in vitro vesicle:vesicle tethering is accompanied by homo-oligomerization of Sro7. (A) Vesicles obtained from *sec6-4* and *sec4-8* mutant strains were generated and used in an in vitro vesicle:vesicle tethering assay in the presence or absence of identical amounts of N-terminally tagged HA-Sro7 and MYC-Sro7. After the addition of mock buffer or DSP cross-linker, the lysates were immunoprecipitated with anti-MYC antibody and subjected to Western blot analysis with anti-HA antibody. (B) Quantitation of Western blots with SD from three independent experiments. *p* value was obtained using a two-tailed Student's *t* test. (C) The Coomassie of the tagged Sro7 proteins used in A. (D) Model for Sro7 as a Sec4 effector that helps to tether post-Golgi vesicles in a pathway that parallels that of the exocyst complex.

simply by the addition of the tagged Sro7 proteins to vesicle membranes—even on vesicles lacking a functional Sec4. This may reflect a role for membrane association as part of the trigger for homo-oligomerization of Sro7. Importantly, though, when the vesicles were incubated under conditions that promoted tethering, we observed robust oligomerization. This strongly suggests that oligomerization of Sro7 is likely to be an important feature of the mechanism by which this protein acts in vesicle tethering.

MATERIALS AND METHODS

Media and reagents

Yeast growth media used in this study include YP composed of 1% bacto-yeast extract and 2% bacto-peptone (Difco), dropout media composed of 0.7% yeast nitrogen base without amino acids (Difco), and synthetic complete amino acid supplement minus appropriate amino acids (US Biological Life Sciences). Dextrose (Fisher Scientific) was used at 2% and raffinose and galactose (from US Biological Life Sciences) were used at 3% and 1%, respectively. Bacteria growth media used in this study include terrific broth composed of 4.7% bacto-TB and 1% glycerol (Fisher Scientific) and lysogeny broth composed of 1% bacto-tryptone (Difco), 0.5% bacto-yeast extract, and 1% sodium chloride (Fisher

Scientific). Agar was obtained from Fisher Scientific.

Reagents used in this study include GTPγS, sodium azide, sodium fluoride, β-mercaptoethanol, pepstatin A, leupeptin, aprotinin, and antipain obtained from Sigma Aldrich and ampicillin, 4-(2-aminoethyl) benzenesulfonyl fluoride, FM4-64, and magnesium chloride obtained from Fisher Scientific. Glutathione agarose and DSP were obtained from Pierce. Dithiothreitol (DTT), bovine serum albumin (BSA), Zymolyase 100T, isopropyl-β-D-thiogalactoside (IPTG), and 5-FOA were obtained from US Biological Life Sciences. Bovine gamma-globulin was obtained from Thermo Scientific. Tween 20 and broad-range protein standards were obtained from Bio-Rad. Secondary antibodies for the Odyssey imaging system are from LI-COR Biosciences and Molecular Probes.

Plasmids used

Plasmids expressing wild-type *SRO7*, *SRO7-K395E*, or *SRO7-R600E* behind a *GAL1/10* promoter were subcloned as *Bam*HI-*Sal*I fragments in pRS313 (*CEN*, *HIS3*). Plasmids for the generation of protein A-tagged forms of Sro7, Sro7-K395E, Sro7-R600E, and Sro7-K395E,R600E were generated by subcloning the wild-type and mutant genes as *Bam*HI-*Hind*III fragments into a vector containing an *ADH1* promoter and a protein A TEV tag(2μ, *URA3*). Plasmids for high copy expression of *SRO7*, *SRO7-K395E*, *SRO7-R600E*, and *SRO7-K395E,R600E* were generated by subcloning the wild-type and mutant genes as *Not*I-*Sal*I fragments into pRS426(*URA3*) and pRS423(*HIS3*). Sro7 with a single *MYC* tag at aa45 was obtained by fusion PCR and subcloned as a *Not*I-*Sal*I fragment in pRS313 (*CEN*, *HIS3*). The *HA* tag was introduced at the same position, following the same subcloning strategy used for the *MYC*-containing construct. Plasmids for the protein purification of the Myc- and HA-containing forms of Sro7 were obtained by subcloning a *Bam*HI-*Hind*III fragment containing the tagged gene in a vector containing an *ADH1* promoter and a protein A TEV tag (2μ, *URA3*).

Protein purification

Sro7, Sro7-K395E, Sro7-R600E, and Sro7-K395E; R600E were obtained using a previously described purification protocol (Rossi et al., 2015). Briefly, an N-terminal Protein A tag containing a tobacco etch virus cleavage site between the tag and the full-length protein allowed release of the Protein A tag after purification with IgG agarose beads. Full-length Sec9 with an N-terminal GST tag and a C-terminal His6 tag was obtained as described previously (Gangar et al., 2005).

Binding assays

Binding assays for Sro7, Sro7-K395E, Sro7-R600E, and Sro7-K395E; R600E to GST, GST-Sec4, and GST-Ypt1 were conducted as previously described (Grosshans et al., 2006).

Vesicle enrichment

Vesicles labeled with the fluorescent protein GFP-Sec4 were obtained from a *sec6-4* secretory mutant strain expressing GFP-Sec4 on a *CEN* plasmid. Briefly, yeast mutant cells were grown overnight in selective media at the permissive temperature of 25°C to early logarithmic growth and then shifted to rich media (YP + 2% glucose) for an hour at 25°C before being placed at the restrictive temperature of 37°C for 2 h to accumulate vesicles. Sodium azide was then added to the culture (20 mM final) and 350 absorbance units of cells were harvested and washed into 10 ml of ice-cold 10 mM Tris, pH 7.5, and 20 mM sodium azide. The cells were then spheroplasted with 10 ml of spheroplast buffer (0.1 M Tris, pH 7.5, 1.2 M sorbitol, 10 mM sodium azide, 21 mM β -mercaptoethanol, and 0.05 mg/ml Zymolyase 100T) for 30 min at 37°C. Spheroplasts were then lysed in 4 ml of ice-cold lysis buffer (10 mM triethanolamine, pH 7.2, and 0.8 M sorbitol) with protease inhibitors (2 μ g/ml leupeptin, 2 μ g/ml aprotinin, 2 μ g/ml antipain, 14 μ g/ml pepstatin A, and 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, HCl). The cell lysate was then centrifuged for 4 min at 450 \times g at 4°C to remove unbroken cells and the remaining supernatant was then centrifuged for 15 min at 30,000 \times g at 4°C in a Sorvall centrifuge to preclarify larger membranes. Approximately 2.5 ml of the resulting supernatant was then layered over 2 ml of an ice-cold sorbitol cushion (20% [wt/vol] sorbitol in 10 mM triethanolamine, pH 7.2) and centrifuged at 100,000 \times g for 1 h at 4°C. The cushion was then aspirated and the vesicle-enriched fraction was resuspended in 300 μ l of lysis buffer and placed on ice for use in the in vitro assay. Vesicles labeled with the lipid dye FM4-64 were obtained from either the *sec6-4* or the *sec4-8* yeast secretory mutant strains with the following adaptations to the vesicle enrichment. In the case of the *sec6-4* secretory mutant strain, the yeast cells were grown overnight in rich media (YP + 2% glucose) at the permissive temperature of 25°C to early logarithmic growth. Mutant yeast cells were then shifted to the restrictive temperature of 37°C for 2 h to accumulate vesicles. Sodium azide was then added to the culture (20 mM final) and 300 absorbance units were then centrifuged and washed into 10 ml of ice-cold 10 mM Tris, pH 7.5, 20 mM sodium azide. The cells were then spheroplasted in 10 ml of spheroplast buffer for 30 mins at 37°C and were then lysed in 4 ml of lysis buffer with protease inhibitors. The lysate was then centrifuged for 4 min at 450 \times g at 4°C to remove unbroken cells and the remaining lysate was centrifuged for 15 min at 30,000 \times g at 4°C in a Sorvall centrifuge. Approximately 2.5 ml of the supernatant was then labeled with FM4-64 (1 μ g/ml) for 10 min on ice. The labeled lysate was then layered over a 2-ml ice-cold sorbitol cushion (20% wt/vol sorbitol in 10 mM triethanolamine) and centrifuged at 100,000 \times g for 1 h at 4°C. The pellet was then resuspended in 600 μ l of lysis buffer. In the case of *sec4-8* vesicles, 600 absorbance units were spheroplasted in 15 ml of spheroplast buffer before lysis in 4 ml of lysis buffer. Vesicles were resuspended in 700 μ l of lysis buffer to obtain similar amount to *sec6-4* vesicles as measured by both Western blot analysis of vesicle marker proteins and negative-stain electron microscopy analysis (Rossi et al., 2015). To demonstrate that clustering was dependent on vesicle accumulation, a wild-type yeast strain, treated identically to *sec6-4* and *sec4-8* mutant strains, was used to generate an FM4-64-labeled fraction, which was examined in the assay in the presence of Sro7 and GTP γ S and found to be completely inactive in cluster formation by fluorescence microscopy (unpublished data).

Asymmetry assay

Vesicles labeled with the lipid dye FM4-64 were obtained from a *sec6-4* or a *sec4-8* mutant strain as previously described (Rossi et al., 2015). GFP-Sec4 labeled vesicles were obtained from a *sec6-4*

mutant strain expressing GFP-Sec4 as previously described (Rossi et al., 2015). Vesicle amounts were monitored by Western blot analysis of the vesicle fractions for Sec4, Snc1/2, and Sso1/2. The in vitro assay was conducted by mixing the FM4-64 vesicles with the GFP-Sec4-labeled vesicles 1:0.5 in the presence of 2 μ M Sro7, 3mM MgCl₂, and 1mMGTP γ S for 30 min at 25°C. "Self"-clustering was monitored by taking images and counting clusters in either FITC or tetramethylrhodamine isothiocyanate (TRITC) channels; "mixed"-clustering was monitored by taking images and counting clusters present in both FITC and TRITC channels. Quantitation of the assay was expressed as the number of vesicle clusters greater than 2 μ m/ μ l for each tethering reaction. The error bar represents the SD from images taken at 60 \times magnification

Cross-linking experiment

Vesicles were obtained from a *sec6-4* mutant strain expressing GFP-Sec4 and from a *sec4-8* mutant strain as described previously (Rossi et al., 2015). The vesicles were incubated with 1.5 μ M MycSro7 and 1.5 μ M HA Sro7 in the presence of 3 mM MgCl₂ and 1 mM GTP γ S for 45 min at 25°C. DSP cross-linker was then added at 0.96 mg/ml on ice for 20 min. Ammonium acetate was then added to 0.22 M for 10 min on ice to quench the reaction. The samples were then heated in 2 \times boiling buffer (2% SDS, 20 mM Tris, pH 8, 10 mM EDTA) at 65°C for 5 min and then diluted with 1.4 ml of IP buffer (10 mM Tris, pH 8, 150 mM NaCl, 0.5% Tween-20, and 0.1 mM EDTA). Samples were centrifuged for 15 min at 4°C and then treated overnight with 75 μ l of 9E10 anti-MYC antibody. Samples were then treated with bridging antibody for 1 h followed by Protein A agarose beads (30 μ l) for 2 h at 4°C. The samples were then washed 4 \times in IP buffer and then heated in SB with 0.1 M DTT at 65°C for 5 min.

Fluorescence microscopy

Yeast strains containing vector control or plasmids (*CEN*, *HIS*) expressing wild-type *SRO7*, *SRO7-K395E*, or *SRO7-R600E* from a *GAL*-inducible promoter were transformed with a plasmid (*CEN*, *LEU*) expressing GFP-Sec4. Strains containing both plasmids were grown overnight to early log phase in selective media containing raffinose (3%) and then induced with galactose (1%) for 6 h before being analyzed by fluorescence microscopy. More than 40 small budded cells were scored for the presence of large clusters of GFP-Sec4 within the mother cell. Quantitation was expressed as the percentage of small budded cells (40 cells were counted) containing a bright patch of GFP-Sec4 within the mother cell.

ACKNOWLEDGMENTS

We thank Melissa Plooster for comments on the manuscript and Keshika Prematilake for technical assistance in this work. This work was supported by National Institutes of Health Grant GM-054712.

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