Yeast Sec1p Functions before and after Vesicle Docking

Kristina Hashizume,* Yi-Shan Cheng,* Jenna L. Hutton,[†] Chi-hua Chiu,[‡]
and Chavela M. Carr*[§]

Departments of *Pathology and Laboratory Medicine and [†]Molecular Genetics and Microbiology, University of Medicine and Dentistry, New Jersey-Robert Wood Johnson Medical School, Piscataway, NJ 08854; and [‡]Department of Genetics, Center for Human Evolutionary Studies, Rutgers University, Piscataway, NJ 08854

Submitted March 2, 2009; Revised August 17, 2009; Accepted September 14, 2009
Monitoring Editor: Patrick J. Brennwald

Sec1/Munc18 (SM) proteins bind cognate soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes and stimulate vesicle membrane fusion. Before fusion, vesicles are docked to specific target membranes. Regulation of vesicle docking is attributed to some but not all SM proteins, suggesting specialization of this earlier function. Yeast Sec1p seems to function only after vesicles are docked and SNARE complexes are assembled. Here, we show that yeast Sec1p is required before and after SNARE complex assembly, in support of general requirements for SM proteins in both vesicle docking and fusion. Two classes of *sec1* mutants were isolated. Class A mutants are tightly blocked in cell growth and secretion at a step before SNARE complex assembly. Class B mutants have a SNARE complex binding defect, with a range in severity of cell growth and secretion defects. Mapping the mutations onto an SM protein structure implicates a peripheral bundle of helices for the early, docking function and a deep groove, opposite the syntaxin-binding cleft on nSec1/Munc-18, for the interaction between Sec1p and the exocytic SNARE complex.

INTRODUCTION

Neurotransmitter secretion, organelle function, and eukaryotic cell growth depend on vesicle fusion reactions regulated by the dynamic interplay between soluble and membrane-anchored protein complexes. Vesicle-anchored (v) and target-membrane-anchored (t) soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) assemble to pin together and catalyze the fusion of vesicles with their target membranes (Weber *et al.*, 1998). Before fusion, soluble Rab GTPases and multicomponent vesicle tethering complexes are required to organize vesicle docking and the subsequent assembly of cognate v-t SNARE complexes (Cai *et al.*, 2007). Sec1/Munc18 (SM) proteins are soluble factors that have been implicated in regulation of vesicle docking, SNARE complex assembly, and vesicle membrane fusion (Jahn, 2000; Burgoyne *et al.*, 2009).

Due to the apparent multiplicity of roles, a coherent model for SM protein function has been difficult to envision. Dramatic vesicle docking defects are observed in the neurons of *Caenorhabditis elegans unc-18* mutants (Weimer *et al.*, 2003), neurons of *Drosophila* *rop* mutants (Schulze *et al.*, 1994), chromaffin cells of Munc18-1 mutants (Voets *et al.*, 2001; Ciufo *et al.*, 2005; de Wit *et al.*, 2006), and vacuoles of yeast *vps33* mutants (Rieder and Emr, 1997). So far, a requirement for yeast Sec1p before vesicle docking at the plasma mem-

brane has not been demonstrated, suggesting specialization of different SM proteins for regulatory functions before SNARE complex assembly.

Only recently has a consensus emerged (Sudhof and Rothman, 2009), confirming the observation in yeast (Carr *et al.*, 1999; Togneri *et al.*, 2006) that SM proteins function as part of the core vesicle fusion machinery, promoting fusion when bound to the assembled SNARE complex (Scott *et al.*, 2004; Dulubova *et al.*, 2007; Shen *et al.*, 2007; Mima *et al.*, 2008). However, no SM protein-SNARE complex structure has been reported, and the mechanism of fusion activation remains to be discovered. The absence of evidence for Sec1p function before vesicle docking may mean the only essential function for SM proteins is stimulation of membrane fusion through interactions with assembled SNARE complexes. Alternatively, there is a general requirement of SM proteins for both vesicle docking and fusion, but docking mutants have not yet been isolated for yeast Sec1p.

Defects in vesicle tethering, docking, and fusion at the yeast plasma membrane are indistinguishable by electron microscopy (Novick and Schekman, 1979; Novick *et al.*, 1980); yet, successful vesicle docking can be determined using an assay for assembled SNARE complexes. With this assay, exocyst mutants, with low or undetectable SNARE complex abundance, were determined to block exocytosis before vesicle docking (Grote *et al.*, 2000). Here, we use random and site-directed mutagenesis to isolate a diverse panel of yeast *sec1* mutants and identify defects in cell growth, SNARE complex assembly, and secretion of cargo carried by dense and light secretory vesicles. Based on these phenotypes, the *sec1* mutants separate into two classes. Class A mutants exhibit a tight block in fusion of both dense and light vesicles and a defect in SNARE complex assembly, suggesting a block before vesicle docking. Class B mutants have SNARE complexes in wild-type abundance, but they are defective in SNARE complex binding. The two classes of mutants cluster in different regions of the SM protein struc-

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09-02-0172>) on September 23, 2009.

[§] Present address: Department of Biochemistry and Biophysics, 300 Olsen Blvd., Texas A&M University, College Station, TX 77843.

Address correspondence to: Chavela M. Carr (chave@tamu.edu).

Abbreviations used: IP, immunoprecipitation; SM, Sec1/Munc18; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor.

Table 1. Yeast strains

Strain	Genotype	Source
CCY6	BY4743, <i>MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 YDR164c::kanMx4/YDR164c (SEC1)</i>	EUROSCARF
CCY32	<i>MATa leu2Δ0 his3Δ1 ura3Δ0 met15Δ0 YDR164c::KANMX pRS416 SEC1</i>	This study
CCY19	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC64 <i>SEC1</i>	This study
CCY28	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC117 <i>sec1-32</i>	This study
CCY29	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC89 <i>sec1-34</i>	This study
CCY44	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC137 <i>sec1-50</i>	This study
CCY45	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC131 <i>sec1-51</i>	This study
CCY46	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC82 <i>sec1-52</i>	This study
CCY50	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC84 <i>sec1-65</i>	This study
CCY51	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC103 <i>sec1-66</i>	This study
CCY52	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC134 <i>sec1-67</i>	This study
CCY53	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC90 <i>sec1-68</i>	This study
CCY55	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC140 <i>sec1-55</i>	This study
CCY57	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC104 <i>sec1-80</i>	This study
CCY58	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC74 <i>sec1-83</i>	This study
CCY59	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC161 <i>sec1-84</i>	This study
CCY64	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC130 <i>sec1-56</i>	This study
CCY65	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC155 <i>sec1-57</i>	This study
CCY66	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC128 <i>sec1-58</i>	This study
CCY67	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC133 <i>sec1-59</i>	This study
CCY71	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC129 <i>sec1-81</i>	This study
CCY91	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC156 <i>sec1-35</i>	This study
CCY92	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC157 <i>sec1-36</i>	This study
CCY94	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC159 <i>sec1-38</i>	This study
CCY95	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC160 <i>sec1-39</i>	This study
CCY100	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC154 <i>sec1-44</i>	This study
CCY102	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC92 <i>sec1-46</i>	This study
CCY104	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC94 <i>sec1-48</i>	This study
CCY105	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC95 <i>sec1-49</i>	This study
CCY106	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC68 <i>sec1-75</i>	This study
CCY107	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC69 <i>sec1-76</i>	This study
CCY108	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC70 <i>sec1-77</i>	This study
CCY109	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC71 <i>sec1-78</i>	This study
CCY110	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC73 <i>sec1-79</i>	This study
CCY162	CCY32, but pRS416 <i>SEC1</i> is replaced with pCC96 <i>sec1-82</i>	This study
CCY42	CCY32 <i>sec18-1</i> , product of a cross with NY432 (P. Novick)	This study
CCY43	CCY42, but pRS416 <i>SEC1</i> is replaced with pCC89 <i>sec1-34</i>	This study
GY3323	<i>MATa leu2-3,112 ura3-52 trp1 his3Δ-200 exo70::HIS3 (CEN TRP1 exo70-38) L-A-</i>	W. Guo
NY778	<i>MATα leu2-3,112 ura3-52 sec6-4</i>	P. Novick
NY784	<i>MATa, leu2-3,112 ura3-52 sec10-2</i>	P. Novick

ture, suggesting a separation of vesicle docking and membrane fusion functions. Thus, like other SM proteins, Sec1p is required both before and after docking, in support of the view that SM proteins use both functions to regulate vesicle membrane fusion.

MATERIALS AND METHODS

Yeast Strains and Media

Strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, yeast cells were grown on yeast peptone dextrose (YPD) media at 25°C (permissive temperature) or 38°C (restrictive temperature). Yeast transformations were performed according to the lithium acetate protocol, as described previously (Gietz and Schiestl, 2007). Sporulation, dissection, and tetrad analysis were performed as described previously (Guthrie and Fink, 1991), by using an Axiophot 20 dissection microscope (Carl Zeiss, Thornwood, NY). Selection was performed on 5-fluoroorotic acid (5FOA) (US Biological, Swampscott, MA) or Synthetic Complete (SC) media lacking leucine (SC-leu) or uracil (SC-ura; MP Biomedicals, Santa Ana, CA), as indicated.

Plasmid and DNA Manipulation

Mutagenesis studies required two centromeric plasmids: a counterselectable *URA3*-marked centromeric (*CEN*) plasmid, pRS416, to create a balancer for the *sec1Δ* null haploid strain; and a *LEU2*-marked *CEN* plasmid, pRS315, to transform the *sec1* mutants into the balanced null strain. To make the balancer

and mutant plasmids, the *SEC1* gene (open reading frame [ORF] plus 745 base pairs upstream and 547 base pairs downstream) was amplified by polymerase chain reaction (PCR) by using YEp24*SEC1* (pCC112; pNB680, Carr *et al.*, 1999) as template. Primers for amplification were BSsec15'G (with BamHI) and BSsec13'J (with XbaI). The amplified 3.4-kb BamHI-*SEC1*-XbaI product was ligated into the two centromeric plasmids (Sikorski and Hieter, 1989; ATCC 67521; American Type Culture Collection, Manassas, VA), creating pRS416*SEC1* (pCC150) and pRS315*SEC1* (pCC64).

To introduce wild-type *SEC1* or *sec1* mutants into the *sec1Δ* genetic background, a heterozygous *sec1Δ* diploid (CCY6; BY4743; EUROSCARF, Frankfurt, Germany) was transformed with pCC150. Transformed diploids were sporulated, and the resulting tetrads were dissected and analyzed on SC-ura plates. The balanced null *sec1Δ URA3* strain (CCY32) was identified by sensitivity to 5FOA, indicating presence of the balancer pCC150. To replace pCC150 with wild-type *SEC1* or *sec1* mutants on a *LEU2* plasmid, CCY32 was transformed with pCC64 or pRS315 containing the *sec1* mutant alleles (for plasmid names, see Table 1). The transformants were selected on SC-leu, and pCC150 was counterselected on 5FOA. The resulting *LEU2 ura3 sec1* strains are listed in Table 1.

Site-directed point mutations were introduced using QuikChange II site-directed mutagenesis kit (Stratagene, Cedar Creek, TX) with pCC64 as a template and polyacrylamide gel electrophoresis (PAGE)-purified oligonucleotide primers (JLH1-10; IDT, Piscataway, NJ), designed with nonoverlapping ends as described previously (Zheng *et al.*, 2004). Conserved sites, identified using the hmmbuild-f algorithm of the HMMER suite of programs (hmmer2.3.2, <http://hmmer.janelia.org>; Hannonhalli and Russell, 2000), were selected for mutagenesis based on amino acid identity in each of the four SM protein families: Sec1, Sly1, Vps33, and Vps45 (Figure 1). "Cleft" mutations

Figure 1. Positions of *sec1* mutations. (A) Sequence conservation among the four SM protein families: Sec1, Vps33, Vps45, and Sly1. Segments of conserved amino acid sequence from *S. cerevisiae* Sec1p (Sce Sec1p) are aligned with the homologous sequence segments from *H. sapiens* Munc-18/nSec1 (Hsa nSec1), Vps33a (Hsa Vps33), Vps45 (Hsa Vps45), and Sly (Hsa Sly1). A capital letter in the consensus sequence (top line) indicates >50% identity. A lowercase letter indicates the highest probability amino acid at that position, and "x" indicates no conservation identified. The asterisks above the consensus sequence indicate conserved sites chosen for mutagenesis. SM protein sequences were separated by phylogenetics (Supplemental Figure S1). Sequences were grouped into four families (Supplemental Table 1), and each group was aligned using the CLUSTALW algorithm in the software package MacVector version 8.1.1. Alignments were evaluated to determine the consensus sequence for all four families (top line), by using the hmmbuild-f algorithm of the HMMER suite of programs (hmmer2.3.2, <http://hmmer.janelia.org>; Hannonhalli and Russell, 2000). (B) Conserved sites chosen for mutagenesis map to buried positions on an SM protein structure. Sites marked by asterisks in A are modeled as spheres (aspartate, red; histidine and arginine, blue) on a ribbon representation of Munc18-1/nSec1: domain 1 (aa 4-134, black), domain 2 (aa 135-245 and 480-592, light gray), and domain 3 (aa 246-479, dark gray). For labeling, amino acid numbers are based on the Munc18-1/nSec1 sequence, with the corresponding yeast numbers in parentheses. Images of nSec1 were created using Pymol (DeLano Scientific, Palo Alto, CA) and Protein Data Bank file 3C98 (Burkhardt *et al.*, 2008), with the syntaxin 1a chain omitted. (C) class A mutations (orange spheres) map to a helical cluster in domain 3b. The positions marked are altered in the randomly generated mutants *sec1-34* [G443E, K494T], *sec1-38* [S391Y, D429G, E446K, I544F], *sec1-39* [M402T, Y437N, I478T], and *sec1-49* [Y437N, I260T] and in conserved-site mutants *sec1-51* [R252A, D255A], *sec1-65* [R252A, E604R], and *sec1-68* [R252A, D307H, H371D]. For labeling, the amino acid numbers are based on the Munc18-1/nSec1 sequence, with the corresponding yeast numbers and mutations in parentheses. Images of nSec1 were created as described in B. (D) Many of the class B mutations (blue spheres) map to a groove between domains 1 and 2. The positions marked are altered in the randomly generated mutants *sec1-32* [L417S, L639S], *sec1-35* [N167I, F221Y, F235L], *sec1-36* [I249N], *sec1-44* [F620S, R643I, L633V, G696S], *sec1-46* [F156S, I187N], *sec1-48* [N81S, R638P]; in conserved-site mutants *sec1-50* [E604A], *sec1-52* [D255A], *sec1-55* [R252L, E604L], *sec1-66* [R252A, H371D], *sec1-67* [D255N]; and in the groove surface mutant *sec1-58* [K79E, Y80E, R169E, K170E]. Images are labeled as described in C. Where the same position has been mutated to more than one amino acid, a comma is used to separate the mutations (i.e., D255A,N). Images were created as described in B.

Table 2. Mutagenesis of yeast *SEC1*

Mutagenesis ^a	Mutation in Sec1p	Growth defect ^b (°C)	Allele
Random	N81S, R638P	38	<i>sec1-48</i>
Random	F156S, I187N	38	<i>sec1-46</i>
Random	N167I, F221Y, F235L	38	<i>sec1-35</i>
Random	I249N	38	<i>sec1-36</i>
Random	S391Y, D429G, E446K, I544F	36	<i>sec1-38</i>
Random	M402T, Y437N, I478T	31	<i>sec1-39</i>
Random	L417S, L639S	38	<i>sec1-32</i>
Random	Y437N, I260T	35	<i>sec1-49</i>
Random	G443E, K494T	31	<i>sec1-34</i>
Random	F620S, R643I, L633V, G696S	38	<i>sec1-44</i>
Conserved	D251A	None	<i>sec1-83</i>
Conserved	D251A, R252A	None	<i>sec1-84</i>
Conserved	R252A	None	<i>sec1-78</i>
Conserved	R252A, D255A	28	<i>sec1-51</i>
Conserved	R252L, D255V, E604L	Lethal	
Conserved	R252A, D307H	None	<i>sec1-80</i>
Conserved	R252A, D307H, H371D	36	<i>sec1-68</i>
Conserved	R252A, H371D	36	<i>sec1-66</i>
Conserved	R252A, E604A	None	<i>sec1-81</i>
Conserved	R252A, E604R	32	<i>sec1-65</i>
Conserved	R252E, E604R	Lethal	
Conserved	R252L, E604L	38	<i>sec1-55</i>
Conserved	E604A	36	<i>sec1-50</i>
Conserved	D255A	37	<i>sec1-52</i>
Conserved	D255N	35	<i>sec1-67</i>
Conserved	D307H	None	<i>sec1-75</i>
Conserved	D307H, H371D	None	<i>sec1-77</i>
Conserved	H371D	None	<i>sec1-76</i>
Groove surface	K79E, Y80E, R169E, K170E	38	<i>sec1-58</i>
Furrow surface	G380R, E381K, D429K	None	<i>sec1-59</i>
Cleft surface	R63D	None	<i>sec1-56</i>
Cleft surface	K64E	None	<i>sec1-82</i>
Cleft surface	R63A, K64A	None	<i>sec1-57</i>
Cleft surface	R63D, K64E	Lethal	
Cleft surface	E291K, E297R, D360R, D362K	None	<i>sec1-79</i>

^a Rationale for mutagenesis.^b Temperature at which growth defect is observed.

were designed based on syntaxin contact sites on the nSec1/Syntaxin 1a structure (Misura *et al.*, 2000). The “furrow” mutant was designed based on clusters of same-charge amino acids that mapped to the surface of a conserved furrow in domain 3 of nSec1 (PDB ID 3C98; Burkhardt *et al.*, 2008), Sly1p (PDB ID 1MQS; Bracher and Weissenhorn, 2002); sSec1 (PDB ID 1EPU, 1FVH; Bracher *et al.*, 2000), and Munc18c (PDB ID 2PJX; Hu *et al.*, 2007). The “groove” mutant was designed to alter the charge property flanking the conserved gap, or groove, between domains 1 and 2. Charged residues were mutated to reverse the charge, unless otherwise indicated (Table 2).

Random mutations were introduced into the *SEC1* open reading frame by using Gene Morph II EZ clone domain mutagenesis kit (Stratagene) with pCC64 as a template. Five pairs of PAGE-purified oligonucleotide primers (JLH1-10; IDT) were designed with nonoverlapping ends. Ten *sec1* mutant plasmid libraries were generated by PCR mutagenesis: two from the entire *SEC1* ORF (PCR 1 and 2), plus two for each of four shorter sequences (PCR 3 and 4, aa 3-132; PCR 5 and 6, aa 133-262; PCR 7 and 8, aa 513-645; and PCR 9 and 10, aa 263-512). The resulting *sec1* mutant plasmid libraries were transformed into CCY32, and transformants were selected on SC-leu. We isolated 2250 colonies that were struck onto 5FOA plates to counterselect the balancer pCC150, and the resulting *ura3-LEU2* colonies were screened for temperature-sensitive (ts) or cold-sensitive (cs) growth defects at 38 and 19°C, respectively. Many of the mutants contained multiple lesions; however, when we attempted to isolate individual point mutations, the ts growth defect was lost. Mutations in the *SEC1* open reading frame were mapped by DNA sequencing (IDT; Table 2).

Immunoprecipitation Assays for SNARE Complex Assembly and Sec1p Binding

Assembled SNARE complexes were assayed by coimmunoprecipitation (IP) of exocytic SNAREs. Wild-type (*SEC1*) and *sec1* mutants were lysed after a 20-min incubation at permissive (25°C) or restrictive (38°C) temperature and analyzed for assembled SNARE complexes, as described (Grote *et al.*, 2000), with the following modifications. An α -Sec9p antibody (Sec9-228; raised against Sec9p, aa 416-651; Covance, Denver, PA) was used to IP Sec9p, an α -Sso1p antibody (Sso1-16,371; gift from M. Munson, University of Massachusetts Medical School, Worcester, MA) was used to detect coprecipitation of Sso1p and an α -Snc2p antibody (Snc2-213, raised against GST-Snc2p, aa 1-93; Covance) to detect Snc2p by Western blot analysis.

Sec1p-binding to SNARE complexes was assayed by co-IP of SNARE complexes with Sec1p. Wild-type (*SEC1*) and *sec1* mutants were lysed after a 20-min incubation at 25 or 38°C, and an α -Sec1p antibody (Sec1-229; raised against Sec1pV5His₆; Covance) was used to IP wild-type and mutant Sec1p. Western blot analysis was used to detect co-IP of Sso1p, which reflects the abundance of assembled exocytic SNARE complexes present in the cell before lysis (Carr *et al.*, 1999). It should be noted that yeast Sec1p does not bind to unassembled Sso1p or the t-SNARE complex of Sec9p and Sso1p (Tognieri *et al.*, 2006). In both IP assays, the abundance of Sso1p in the input lysates was detected by Western blot analysis by using α -Sso1p (Sso1-16,371).

Invertase and Bgl2p Secretion Assay

Invertase assays were performed as described previously (Wiederkehr *et al.*, 2003). In brief, *SEC1* and *sec1* mutants were shifted to 38°C for 1 h in low glucose medium (0.1%). Total and external invertase activities were measured using an enzyme assay (Goldstein and Lampen, 1975). Internal invertase was determined by subtracting the external invertase activity from the total. The average percentage of internal invertase was determined from four independent experiments, unless otherwise indicated.

Internal Bgl2p accumulation was determined as described previously (Harsay and Schekman, 2007). In brief, yeast strains were grown at 25 or 38°C for 1 h. Cells were spheroplasted and internal Bgl2p was assayed by Western blot, by using an α -Bgl2p antibody (gift from Edina Harsay, University of Kansas, Lawrence, KS). An α -alcohol dehydrogenase (ADH) antibody (Abcam, Cambridge, MA) was used to detect ADH, which was used as an internal loading control. The intensity of the Bgl2p bands was normalized with the ADH band intensity and quantified by ChemiDocXRS and Quantity One, version 4.4.1 (Bio-Rad Laboratories, Hercules, CA). The average internal Bgl2p intensity was determined from three independent experiments, unless otherwise indicated.

Bud Emergence Assay

Cultures of early log phase yeast cells were arrested at G1 by addition of 6 μ M α -factor (Sigma-Aldrich, St. Louis, MO) at 25°C. The cells were washed with fresh YPD medium to remove the α -factor and incubated for 60 min at the indicated restrictive temperatures. For each strain studied, the bud size was measured using IP Lab Image Analysis software (BioVision Technologies, Exton, PA), with images captured by differential interference contrast (DIC) microscopy. For each strain, 200 cells in total were counted. Daughter cells $\leq 2 \mu$ m in diameter were counted as small buds, and daughter cells $> 2 \mu$ m were counted as large buds.

SNARE Complex Binding by Immobilized Sec1p-V5-His₆

His-tagged Sec1p (Sec1p-V5-His₆), Sec1-34p, or Sec1-58p was expressed in yeast by using pYES2/CT plasmid transformed into the *Saccharomyces cerevisiae* strain INVSc1, as described by the manufacturer (Invitrogen, Carlsbad, CA). After induction with 2% galactose, cells were harvested and resuspended in wash buffer (50 mM HEPES, pH 7.4, 20 mM NaF, and 20 mM Na₃PO₄). Washed cells were lysed in binding buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% Igepal, 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and yeast protease inhibitor mixture [Complete; Roche Applied Science, Indianapolis, IN]). The lysate was clarified by sequential centrifugation at 15,000 $\times$ g and 100,000 $\times$ g for 30 min each. To standardize the amount of Sec1p-V5-His₆ used for the binding reactions, 2 μ g of V5 monoclonal antibody (Invitrogen) was used to purify and immobilize Sec1p-V5-His₆ on protein G-Sepharose resin (Invitrogen). Excess Sec1p-V5-His₆ and cellular proteins were washed away with binding buffer. Before addition to the binding reaction, SNARE complexes or Sso1p/Sec9CT binary complexes were assembled as described previously (Nicholson *et al.*, 1998). Binary or ternary SNARE complexes (0.3 or 1 μ M) were added to immobilized Sec1p-V5-His₆, or mutant proteins, and incubated for 2 h at 4°C. After three washes with binding buffer, bound proteins were detected by SDS-PAGE analysis and stained with Coomassie Blue R250.

RESULTS

Sec1p interacts specifically with the exocytic SNARE complex (Carr *et al.*, 1999; Tognieri *et al.*, 2006), and the SM protein-SNARE complex interaction has been shown to

stimulate vesicle fusion (Scott *et al.*, 2004; Dulubova *et al.*, 2007; Shen *et al.*, 2007; Mima *et al.*, 2008). There is also evidence that SM proteins may function upstream of vesicle fusion, through interactions with vesicle tethering machinery (Sato *et al.*, 2000; Seals *et al.*, 2000; Wiederkehr *et al.*, 2004; Wagner *et al.*, 2006; Peplowska *et al.*, 2007). However, it is unknown whether these interactions represent a general function of SM proteins. To better clarify the role or roles of Sec1p, we used site-directed and random mutagenesis to generate a large panel of *sec1* mutants.

Isolation of *sec1* Alleles

Sites with a high percentage of amino-acid identity among SM protein sequences were targeted for mutagenesis of the yeast *SEC1* gene. From 37 taxa, 164 unique sequences were identified with similarity to Sec1, Sly1, Vps33, or Vps45 from yeast (*S. cerevisiae*) and human (*Homo sapiens*). Sequences were sorted into four families (Sec1, Sly1, Vps33, and Vps45; Supplemental Table S1) by using a phylogenetic approach similar to those used in other studies of SM protein family evolution (Supplemental Figure S1; Arac *et al.*, 2005; Koumandou *et al.*, 2007). Based on these results, a separate multiple sequence alignment was made for each family, by using the CLUSTALW algorithm in the software package MacVector version 8.1.1 under default settings. The four alignments were used as input for Protein Family Alignment Annotation Tool (Johnson *et al.*, 2003), to determine percentage of identity at each amino acid position for each family. The four alignments were also used as input for the HMMER algorithm hmmbuild-f (Hannenhalli and Russell, 2000), to determine a consensus sequence for each family. The four consensus sequences were aligned and hmmbuild-f was used a second time to determine a consensus among all four families (Figure 1A).

Charged residues occupy many of the most highly conserved sites. Therefore, sites that were >95% conserved by charge were chosen for mutagenesis. Mapping the conserved sites onto the nSec1/Munc18 structure revealed two charge pairs (Figure 1B): the R235/E549 (R252/E604 in yeast Sec1p) salt bridge buried at the protein core, and D285/H347 (D307/H371) located in domain 3a. D234 (D251) seems to be involved in backbone hydrogen bonds in the core of domain 2. Another conserved aspartate, D238 (D255), forms hydrogen bonds with a short helical turn (D238-V243 in nSec1/Munc18), and with R235, interactions observed in all SM protein crystal structures resolved to date (Bracher *et al.*, 2000; Misura *et al.*, 2000; Bracher and Weissenhorn, 2002; Hu *et al.*, 2007). Chosen sites were substituted with alanine or leucine, to replace the charges with neutral or hydrophobic residues (Table 2). Pairs of charges were also reversed, to determine the effect of conserving the bond, but with reversed topology. Aspartate, D238 (D255) was substituted with alanine, or asparagine, a favored N-cap residue (Aurora and Rose, 1998), to test its possible function as a helix-capping residue. Of the 18 conserved-site mutants studied, eight exhibited a ts growth defect, two were lethal and eight showed no growth defect at either 38 or 19°C (Table 2).

Random mutations were introduced into the *SEC1* coding sequence by PCR mutagenesis (see *Materials and Methods*). From 10 *sec1* mutant plasmid libraries, each created by an independent PCR reaction, 2250 transformants in total were screened. Of these, 10 exhibited ts growth defects upon loss of the *SEC1* balancer plasmid. The fact that none of the conserved-site mutations was isolated from this screen indicates that the random mutagenesis approach we used was not saturating.

In total 19 mutants with a recessive, ts growth defect were isolated from the combined random and site-directed mutagenesis approaches, including an additional surface mutation, described below. The positions of the mutations were determined by DNA sequencing of the *SEC1* open reading frame (IDT; Table 2) and mapped on the nSec1/Munc18 structure (Figure 1). Although we also screened for conditional viability at 19°C, no cold-sensitive mutants were isolated. Because *SEC1* is an essential gene, mutations that disrupt folding or abolish an essential binding interaction are expected to be lethal at all temperatures and therefore missed by the random mutagenesis screen. However, three site-directed mutants were determined to be lethal upon replacement of the wild-type gene with the *sec1* mutant allele (Table 2).

Ts mutants could be defective due to a weakened binding interaction, or a temperature-sensitive folding defect. A previous study found increased degradation at restrictive temperature (Brummer *et al.*, 2001) in the *sec1-11* mutant (Novick and Schekman, 1979), suggesting a folding defect in that ts *sec1* allele. In another study, we showed that recombinant, wild-type Sec1p produced in *Escherichia coli* was insoluble, with no detectable Sec1p in the supernatant fraction after centrifugation at $300,000 \times g$ (Togneri *et al.*, 2006), suggesting aggregation due to misfolding. Wild-type Sec1p in yeast lysates separates into a particulate and a soluble fraction: the soluble form is monomeric and binds specifically to SNARE complexes (Togneri *et al.*, 2006). Although it is possible to have local folding defects that do not exhibit increased degradation or aggregation, we used these criteria to test for a temperature-sensitive, global folding defect that might explain a loss of Sec1p function in *sec1* mutants. The stability and solubility of the mutant proteins were determined to be comparable with Sec1p from an isogenic wild-type strain (Supplemental Figure S2).

Two Classes of *sec1* Mutants

Based on the reported functions of other SM proteins for both vesicle docking and fusion, we expected that the isolation of a large panel of *sec1* mutants would reveal 1) whether there is a docking role for Sec1p and 2) the surface used by Sec1p to bind the exocytic SNARE complex for activation of vesicle membrane fusion. To determine whether we had isolated separate functions for Sec1p, we screened the 20 loss-of-function *sec1* mutants for SNARE complex assembly (an indication of vesicle docking) and SNARE complex binding, and we found that the mutants fall into two functionally distinct classes: class A and class B (Figure 2).

Class A mutants are defective in SNARE complex assembly. Assembled SNARE complexes were detected using a co-IP assay (Togneri *et al.*, 2006). A Sec9p IP was used to screen the *sec1* mutants for coprecipitation of Sso1p (Figure 2A, top). Yeast exocytic SNARE complexes assemble *in vivo* and do not continue to assemble or exchange in lysates prepared under ATP-depleting conditions (Carr *et al.*, 1999). Thus, the intensity of the Sso1p band detected by Western blot analysis reflects the abundance of assembled SNARE complexes in the cell at the time of lysis. In class A mutants, such as *sec1-34* and *sec1-68*, the abundance of SNARE complexes was significantly diminished after a 20-min shift to the restrictive temperature. Diminished levels of assembled SNARE complexes in the class A mutants suggests a block before vesicle docking, a phenotype observed previously for mutants of the exocyst tethering complex, such as *sec6-4* (Figure 2A; Grote *et al.*, 2000).

Class B mutants have a SNARE-complex binding defect, despite wild-type levels of SNARE complexes. The Sec1p–

Figure 2. Phenotypes of class A and class B *sec1* mutants. (A) SNARE complex abundance is diminished in class A mutants, whereas SNARE complex binding to Sec1p is disrupted in class B mutants. An α -Sec9p antibody (Sec9-288) was used to IP Sec9p in an assay for assembled SNARE complexes (top). An α -Sec1p antibody (Sec1-229) was used to IP Sec1p in an assay for Sec1p-binding to SNARE complexes (middle). Coprecipitation of Sso1p with either Sec9p or Sec1p was detected by Western blot analysis by using an α -Sso1p antibody (Sso1-16,371). Wild-type (SEC1) and the indicated *sec1*, *sec6*, and *exo70* mutant yeast strains were lysed after a 20-min shift from permissive (25°C) to restrictive (38°C) temperature. Protein concentration in the lysates was determined using the Bradford assay (Bio-Rad Laboratories). Bound proteins were separated by SDS PAGE, and 0.75% of the lysates used for the IPs is shown (Input, bottom). (B) Both invertase and Bgl2p secretion are tightly blocked in class A mutants and blocked with a range of severity in class B mutants. Accumulation

of invertase and Bgl2p was measured in wild-type (SEC1) and the indicated mutant yeast strains after a 60-min shift to the restrictive temperature (38°C). Invertase activity was measured using an enzyme assay, and the average percentage of internal invertase ($\pm$ SEM; $n = 4$) was plotted on a bar graph (black; see also Supplemental Figure S2). The average internal Bgl2p was measured by Western blot analysis by using an α -Bgl2p antibody (gray; for Westerns, see Supplemental Figure S2). The averaged units of intensity plotted ($\pm$ SEM; $n = 3$) represent the optical density per square millimeter of each Bgl2p band, normalized to the intensity of internal loading control (ADH).

SNARE complex interaction is detected in a binding assay (Figure 2A, middle), by using an α -Sec1p antibody (Sec1-229) to IP wild-type or mutant Sec1p (Supplemental Figure S3). Yeast Sec1p binds specifically to the assembled exocytic SNARE complex, composed of Sso1p, Sec9p, and Snc2p, and not to the t-SNARE complex (Sso1p and Sec9p), or the individual SNAREs (Tognieri *et al.*, 2006). Therefore, the Sec1p IP was used to screen the *sec1* mutants for SNARE complex binding. In this assay, the Sso1p band on the Western blot reflects the fraction of SNARE complexes coprecipitating with Sec1p (Carr *et al.*, 1999; Tognieri *et al.*, 2006). SNARE complexes coprecipitating with Sec1p from mutant strains were significantly diminished, compared with an isogenic wild-type control (Figure 2A, middle). Reduced Sso1p coprecipitation with Sec1p from class A mutants is probably due to the diminished levels of assembled SNARE complexes in those strains (Figure 2A, top). For class B mutants, SNARE complexes are abundant (Figure 2A, top; coprecipitation of Snc2p and Sso1p with Sec9p, Supplemental Figure S3), suggesting diminished coprecipitation of Sso1p is due to a SNARE-complex binding defect in mutant Sec1p protein. By contrast, neither SNARE complex assembly nor Sec1p binding was defective in the exocyst mutant *exo70-38* (Figure 2A), suggesting the ts defect in this exocytosis mutant is independent of SNARE complex assembly or Sec1p binding.

Secretory Phenotypes of Class A and B Mutants

Electron micrographs of thin sections of yeast exocytosis mutants reveal gross accumulation of secretory vesicles after exposure to the restrictive temperature (Novick and Schekman, 1979; Novick *et al.*, 1980). The secretory vesicles that accumulate in exocytosis mutants, such as *sec6-4*, separate into two peaks on density gradients (Harsay and Bretscher, 1995): dense vesicles, which carry the enzyme invertase to the plasma membrane via the endosome, and the more abundant light vesicles, which carry cell wall components, such as the endoglucanase Bgl2p.

To determine the types of secretory vesicles that accumulate in the class A and class B *sec1* mutants, the intracellular levels of invertase and Bgl2p were monitored using established assays (see *Materials and Methods*). In general, both classes of mutants exhibited higher intracellular Bgl2p levels compared with the isogenic wild-type control strain and resembled the exocyst mutant *sec6-4* (Figure 2B, gray bars; for Bgl2p Western blots of all mutants, see Supplemental Figure S4A; Harsay and Bretscher, 1995; He *et al.*, 2007). In contrast to the Bgl2p phenotype, a strong block in invertase secretion was found to be a hallmark of class A mutants (Figure 2B, black bars; all mutants, Supplemental Figure S4B). Class A mutants uniformly retained $>90\%$ of the invertase enzyme, similar to that observed for the exocyst mutants, *sec6-4* (Novick *et al.*, 1980). However, in class B mutants, the invertase phenotypes ranged from a strong intracellular accumulation, as in *sec1-67* ($>90\%$), to very weak or no significant increase in invertase accumulation, as in *sec1-50* ($<25\%$; Figure 2B and Supplemental Figure S4B).

Bud Emergence Is Blocked in a Subset of *sec1* Mutants

Studies of *exo70-35* and *exo70-38*, and the secretion-specific allele, *cdc42-6*, draw a connection between a block in Bgl2p secretion (without an invertase secretion defect) and delayed bud emergence, suggesting a link between these genes and the cell cycle (He *et al.*, 2007; Adamo *et al.*, 2001). Given that a subset of class B mutants exhibited a similar secretion phenotype, *sec1* mutants were examined for a possible delay in bud emergence (Figure 3). *MATa* strains of *sec1* mutants were arrested in G1 by exposure to α -factor and then washed with fresh media to initiate growth. After 60 min at the restrictive temperature, the majority of wild-type cells had produced large buds ($>65\%$), with few unbudded cells (10%). Class A mutants, such as *sec1-68*, exhibited very few cells with buds (3%), whereas most cells remained in the unbudded stage (97%), resembling the exocyst mutant *sec10-2* ($>96\%$ unbudded). Class B mutants exhibited a range of budding defects. Mutants tightly blocked in both

Figure 3. Growth of the daughter cell is tightly blocked in class A mutants. Wild-type (*SEC1*) and the indicated *sec1* and *sec10* mutant cells were synchronized using α -factor. Growth of the daughter cell (bud) was initiated at restrictive temperature (38°C) by washing out the α -factor with fresh media. Images were taken at 0 and 60 min, by using DIC microscopy and analyzed using IP Lab Image Analysis software (BioVision Technologies). The percentage of small-budded cells (arrowheads; $<2 \mu\text{m}$; gray bars), large-budded cells (arrows; $>2 \mu\text{m}$; white bars), and unbudded cells (black bars) were quantified for the 60-min time point and plotted as a bar graph ($n = 200$).

Bgl2p and invertase secretion, such as *sec1-67*, exhibited a severe budding defect (99% unbudded). In *sec1-58*, an allele with a partial block in invertase secretion (Supplemental Figure S4B), ~50% of the cells remained unbudded. Surprisingly, no discernible budding defect was observed for *sec1-50*, an allele that resembles *exo70-35*, *exo70-38*, and *cdc42-6* in its secretion phenotype. Using growth curves, we determined that the onset of the temperature-sensitive growth defect observed for *sec1-50* occurs after the first doubling (data not shown); thus, in this case, growth arrest is not due to a bud emergence defect. In general, there seems to be a correlation between the severity of the invertase secretion block and the inability of *sec1* mutants to produce buds at restrictive temperatures.

A Distinct Function for Sec1p Upstream of SNARE Complexes

The differences observed between *sec1* mutants could be explained trivially, if a common function were disrupted, but more severely in class A than in class B mutants. Like class B mutants, coprecipitation of SNARE complexes with the mutant Sec1p is strongly diminished in class A mutants (Sec1p IP, Figure 4A). SNARE complex abundance is also severely reduced in class A mutants, with little Sso1p copre-

cipitation in the Sec9p IP assay (Sec9p IP, Figure 4A). By contrast, the assay for assembled SNARE complexes revealed wild-type coprecipitation of Sso1p from class B mutants (Sec9p IP, Figure 2A), including *sec1-67*, with an invertase secretion defect as strong as the class A mutants (Figure 2B and Supplemental Figure S4).

We considered the possibility that lower SNARE complex abundance in class A mutants could be due to a decrease in the overall abundance of one or all of the exocytic SNAREs: Sec9p, Sso1p, or Snc2p. Decreased SNARE abundance has been observed previously for other SM protein mutants such as *vps45 Δ* , which has diminished levels of the syntaxin SNARE, Tlg2p in the absence of Vps45p (Bryant and James, 2001). Western blot analysis of Sec9p, Sso1p and Snc2p indicated that all three exocytic SNAREs are present in wild-type abundance after a shift to the restrictive temperature (Figure 4B), indicating decreased SNARE protein concentration is not the cause of the low abundance of SNARE complexes in the class A mutants.

We reasoned that if the function lost in class A mutants is distinct from class B mutants, Sec1p from a class A mutant should have detectable SNARE complex binding activity. If SNARE complexes could be restored in a class A *sec1* mutant, the mutant Sec1p could be tested for the SNARE com-

Figure 4. A function distinct and before the SNARE complex binding activity of Sec1p is defective in class A mutants. (A) Reduced abundance of SNARE complexes is observed in all class A *sec1* mutants. Representative Sso1p Western blots indicate the results of the assays for assembled SNARE complexes (αSec9p; top) and for Sec1p-binding to SNARE complexes (αSec1p; middle), as described in Figure 2A. Bound proteins were separated by SDS PAGE, and 0.75% of the lysates used for the IPs are shown (Input). (B) Sec9p, Sso1p, and Snc2p are present at wild-type abundance in class A *sec1* mutants. Wild-type (*SEC1*) and *sec1* mutants were lysed after 20-min incubation at the restrictive (38°C) temperature. For each lysate, 10 μg of total protein was loaded and separated by SDS-PAGE. SNAREs were detected by Western blot analysis by using α-Sec9p, α-Sso1p, and α-Snc2p (Snc2-213). The *sec1-65* sample was run on a separate gel, which did not resolve the doublet characteristic of Sec9p. (C) Rapid accumulation of SNARE complexes in *sec18-1* increases the abundance of and binding to SNARE complexes by Sec1-34p. The results of the SNARE complex assembly (αSec9p) and binding (αSec1p) IP assays are shown for wild-type (*SEC1* *SEC18*), *sec18-1* (*SEC1* *sec18-1*), *sec1-34* (*sec1-34* *SEC18*), and the double mutant *sec1-34* *sec18-1*. Cells were lysed after 20-min incubation at the restrictive (38°C) temperature. The Western blot also indicates the relative abundance of Sso1p and Snc2p in 0.75% of the lysates used for the IPs, as shown in Figure 2A. (D) High-copy expression of *SEC9* restores growth and partially restores SNARE complex assembly and mutant Sec1p binding to SNARE complexes in the class A mutant *sec1-34*. On an SC-leu-ura plate incubated at restrictive temperature (32°C), growth of the

following transformants is shown in triplicate: wild-type (*SEC1*) with 2μ *SEC9*, *sec1-34* with a control vector and *sec1-34* with 2μ *SEC9* (top). Bottom, results of the SNARE complex assembly (αSec9p) and binding (αSec1p) IP assays, performed as described in Figure 2A. Also indicated on the Western blot is the abundance of Sec9p (overexpressed in the strains transformed with 2μ *SEC9*), Sso1p and Sncp in 0.75% of the lysates used as inputs for the IP assays.

plex binding function using the Sec1p IP assay. By crossing class A mutant *sec1-34* with the SNARE complex disassembly mutant *sec18-1*, we isolated a double mutant, *sec1-34 sec18-1*. With this mutant, we could take advantage of the rapid onset of SNARE complex accumulation in *sec18-1* (Grote *et al.*, 2000) in the *sec1-34* background at restrictive temperature (38°C; Sec9p IP, Figure 4C). Although diminished relative to the *sec18-1* single mutant, SNARE complexes are detectable at much greater abundance in the *sec1-34 sec18-1* double mutant than in the *sec1-34* single mutant. Furthermore, a small fraction of the SNARE complexes that persist in *sec1-34 sec18-1* at restrictive temperature, although nonfunctional for restoring growth, bind to and coprecipitate with the mutant Sec1-34p protein (Sec1p IP, Figure 4C).

SNARE complex abundance and Sec1p binding are also restored in *sec1-34* by an excess of Sec9p. The *sec1-34* ts growth phenotype is suppressed by high-copy expression of Sec9p, by using a 2μ *SEC9* plasmid, and increased SNARE complex abundance and binding is observed using the IP assays (Figure 4D). Together with the *sec1-34 sec18-1* double mutant results, these results suggest that class A mutants have a severe block in secretion, not because the mutant

Sec1p protein is unfolded, nor due to a defect in SNARE-complex binding, but probably due to a deficiency in a function required upstream of vesicle docking and SNARE complex assembly.

Conserved sites are buried and may play a role in SM protein structure and dynamics that is required for both vesicle docking and SNARE-complex binding functions. In contrast to the class A and class B mutants isolated from the random mutagenesis screen, which map to distinct regions of the SM protein structure (Figure 1), conserved-site mutations are found in both class A and class B, as might be expected if the two essential functions are conserved. Charge pairs, such as those in the conserved salt bridge formed by R235 and E549, are not necessarily classified together (also true for Sly1p, Li *et al.*, 2005), as would be expected if a single function were disrupted by a point mutation in either one of the pair. The salt bridge per se is not essential: changing both R235 and E549 simultaneously to the charge-neutral amino acid alanine (*sec1-51*, R252A, E604A; Table 2) resulted in wild-type growth. However, changing them instead to leucines (*sec1-55*) resulted in a class B phenotype (Supplemental Figure S4), suggesting

packing and/or flexibility at this position is important for a SNARE complex interaction.

Our analysis of conserved sites has also uncovered a role for an invariant aspartate, D238 in nSec1/Munc18-1. Unlike a nearby aspartate, D234 (D251 in yeast), which tolerates alanine substitution (i.e., *sec1-83* and *sec1-84*), D238 (D255) is sensitive to mutation. D238 appears in the structures to be acting as an N-cap, stabilizing a short helical turn (residues 238–243) through H-bonds with the side chain carboxylate. Replacement of D238 with alanine (*sec1-52*, D255A), which

cannot make the capping H-bonds, resulted in a temperature-sensitive growth defect and a class B secretory phenotype (Supplemental Figure S4). However, despite its favorability to form an N-cap (Aurora and Rose, 1998), asparagine at that position has an even more dramatic secretory block (*sec1-67*, D255N), indicating that an N-cap cannot be the sole conserved function. This invariant aspartate may also be required as a charge partner for R235 during SM protein folding or for conformational changes required for the vesicle docking and/or membrane fusion mechanisms.

Figure 5. Mutations in a conserved groove disrupt Sec1p binding to the SNARE complex. (A) The positions of surface-directed mutations are mapped on nSec1. Mutations were introduced at three surfaces, indicated with dashed lines: the cleft, *sec1-79* [E291K, E297R, D360R, D362K] (green spheres) and pC72 [R63D, K64E] (black spheres); the furrow, *sec1-59* [G380R, E381K, D429K] (yellow spheres); and the groove *sec1-58* [K79E, Y80E, R169E, K170E] (blue spheres). The box (left; ribbon representation) highlights the groove region, where amino acids (blue spheres) are altered in class B mutants *sec1-35* [N167I, F221Y, F235L], *sec1-36* [I249N], *sec1-46* [F156S, I187N], and *sec1-48* [N81S, R638P] (see Table 2) and the site-directed groove surface mutant *sec1-58* [K79E, Y80E, R169E, K170E] (bold type). For labeling, amino acid numbers are based on the Munc18-1/nSec1 sequence, with the corresponding yeast numbers and mutations in parentheses. Images were created as described in Figure 1. (B) Groove mutants have a ts growth defect. Wild-type (*SEC1*) and *sec1* mutants of the cleft (*sec1-79*), furrow (*sec1-59*), and groove (*sec1-35*, *sec1-36*, *sec1-46*, *sec1-48*, and *sec1-58*) were tested for growth at 25 and 38°C. A yeast strain bearing the plasmid pC72 [R63D, K64E] in place of *SEC1* was inviable. (C) Sec1p from the groove mutants binds weakly to assembled SNARE complexes. Wild-type (*SEC1*) and ts *sec1* mutants (*sec1-35*, *sec1-36*, *sec1-46*, *sec1-48*, and *sec1-58*) were assayed for assembled SNARE complexes (top) and SNARE complex binding (middle), as described in Figure 2A. Input (bottom) represents 0.75% of the lysate used for the IPs.

Mapping the SNARE Complex Binding Surface on Sec1p

Mutations that affect the Sec1p-SNARE complex interaction are expected to map to binding surfaces or to buried core positions that stabilize the SNARE-complex binding conformation of Sec1p. The crystal structures of nSec1/Munc18 (Misura *et al.*, 2000), sSec1 (Bracher *et al.*, 2000), Sly1p (Bracher and Weissenhorn, 2002), and Munc18c (Hu *et al.*, 2007) have three defined channels that are connected to each other and are large enough to accommodate part or all of a SNARE complex (Figure 5A). An area described as the cleft is formed between domains 1 and 3 where closed conformation of Syntaxin 1a binds to nSec1/Munc18 (Misura *et al.*, 2000). The groove describes the channel formed between domains 1 and 2, opposite of the syntaxin-binding cleft. Close to the base of the molecule is the furrow, flanked by domain 3a and 3b.

Using the nSec1/Munc18 crystal structure as a guide, mutations were designed and introduced onto the surface of the cleft, groove and furrow of Sec1p, in an attempt to disrupt interactions with the exocytic SNARE complex. Clusters of same-charged surface residues in yeast Sec1p were reversed to the opposite charge by site-directed mutagenesis (Figure 5A). No growth defect was observed for mutations introduced in the furrow (*sec1-59*) or cleft (*sec1-79*; Figure 5B), with the exception of the cleft mutations at R65/E66 (yeast Sec1p R63D, K64E), which is a recessive lethal mutation. By contrast, directed mutagenesis in the groove resulted in a mutant, *sec1-58*, with a temperature-sensitive growth defect (Figure 5B). The mutant exhibited wild-type abundance of SNARE complex, with a strong defect in Sec1p binding to SNARE complexes (Figure 5C). The binding defect observed in *sec1-58* was similar to that observed for other class B mutants, *sec1-35*, *sec1-36*, *sec1-46*, and *sec1-48*, which cluster in the groove area (Figure 5, A and C). Thus, the results of both site-directed and random mutagenesis converge on a groove between domains 1 and 2 of Sec1p as a surface that, although not necessary for SNARE complex assembly, seems to be important for SNARE complex binding to Sec1p.

An *in vitro* binding assay with purified, soluble SNAREs (Togneri *et al.*, 2006) was used to further examine the SNARE-complex binding defect in the designed groove mutant *sec1-58*. Cytoplasmic SNARE complexes were incubated with Sec1p modified at the C terminus with a V5 epitope and a six-histidine tag for purification (Figure 6). Wild-type Sec1p-V5-His₆ binds ternary, cytoplasmic SNARE complexes with an apparent binding constant of 300 nM (Togneri *et al.*, 2006). At this concentration of cytoplasmic SNARE complexes, we detected weak binding to immobilized Sec1-58p-V5-His₆, compared with either wild-type Sec1p-V5-His₆, or the class A mutant, Sec1-34p-V5-His₆. SNARE complex binding is specific, with no detectable binding to binary t-SNARE complexes (first three lanes, Figure 6), and requires the SNARE-motif linker region of Sec9p (Supplemental Figure S5), as has been demonstrated previously for wild-type Sec1p (Togneri *et al.*, 2006).

The Sec1p from class A mutant *sec1-34* binds to SNARE complexes accumulated *in vivo* (Figure 4, C and D) and Sec1-34p-V5-His₆ binds to purified cytoplasmic SNARE complexes, *in vitro* (Figure 6), albeit with slightly weaker affinity than the wild-type protein. Thus, the severe secretion and growth phenotypes observed for class A mutants are unlikely to be attributed to a severe SNARE complex binding defect. By contrast, the binding defect observed *in vivo* for class B mutants, such as *sec1-58*, is also observed *in vitro*, with purified soluble proteins, implicating the groove sur-

Figure 6. *In vitro* binding between the SNARE complex and mutant Sec1p. Sec1p-V5-His₆ protein from wild-type (*SEC1*), class A mutant *sec1-34*, and class B mutant *sec1-58* was affinity purified from yeast lysates by using an α -V5 antibody and immobilized on protein G-Sepharose resin for binding studies. The cytoplasmic domains of binary (Sso1p[1-265] and Sec9[416-651]) or ternary (Sso1p[1-265], Sec9p[416-651], and Snc2p[1-93]) SNARE complexes were incubated at 300 nM with immobilized mutant and wild-type Sec1p, as described previously (Togneri *et al.*, 2006). Bound Sec1p-V5-His₆ and SNAREs were resolved by SDS-PAGE and stained using Coomassie Blue R250. Sec1-58p-V5-His₆ migrates slightly slower than Sec1p-V5-His₆ from the other strains. An asterisk marks a band likely to be a degradation product of Sec1p-V5-His₆ that copurify with the protein from the mutant strains. At higher SNARE complex concentrations, but not observed in this gel, the Snc2p band is detected at the position marked (Snc2p[1-93]; see Supplemental Figure S5). A diffuse band detected slightly above the Snc2p[1-93] position is a contaminant. V5 antibody heavy chain (Hc) and light (Lc) are also indicated. The positions of molecular weight standards are indicated in kilodaltons.

face as an important interaction site between Sec1p and exocytic SNARE complexes.

DISCUSSION

Using random and site-directed mutagenesis, we have identified two classes of *sec1* mutants that suggest 1) a requirement for SM proteins before vesicle docking extends to yeast Sec1p and 2) a groove opposite the syntaxin-binding cleft is necessary for Sec1p interaction with the exocytic SNARE complex. These findings help to bridge a gap between Sec1p, which specifically binds to assembled SNARE complexes (Togneri *et al.*, 2006), after vesicle docking (Grote *et al.*, 2000), and other SM proteins, which have a high affinity for the unassembled syntaxin SNARE and function before vesicle docking (Munson and Bryant, 2009). Despite different modes of SNARE interactions (Gallwitz and Jahn, 2003), there is a growing consensus that SM proteins are required to activate SNARE complexes for membrane fusion (Scott *et al.*, 2004; Dulubova *et al.*, 2007; Shen *et al.*, 2007; Starai *et al.*, 2008). It is less clear whether every vesicle docking reaction requires an SM protein and what function the SM protein provides to ensure specific vesicle attachment and SNARE complex assembly.

The mechanism used by SM proteins to regulate vesicle docking may have evolved to match the different require-

ments of regulated vesicle fusion, for example, exocytosis in neuroendocrine cells versus the more constitutive vesicle fusion required for yeast cell budding. In chromaffin cells, Munc18-1 binds the syntaxin N-peptide domain to inhibit synaptic SNARE complex assembly until the N-peptide domain is displaced (Burkhardt *et al.*, 2008). The syntaxin N-peptide binding mode is widespread among the SM proteins, including yeast Sly1p and mammalian Sly1 (Yamaguchi *et al.*, 2002), yeast Vps45p and mammalian Vps45 (Dulubova *et al.*, 2002; Carpp *et al.*, 2006), worm unc-18 (Johnson *et al.*, 2009), Munc18-1 (Dulubova *et al.*, 2007; Khvotchev *et al.*, 2007; Burkhardt *et al.*, 2008), and Munc18c (Latham *et al.*, 2006), and it has been proposed as part of a general mechanism for regulation of vesicle docking (Shen *et al.*, 2007; Burkhardt *et al.*, 2008; Johnson *et al.*, 2009). However, the syntaxin N-peptide interaction is dispensable for the function of Sly1p (Peng and Gallwitz, 2004), Vps45p (Carpp *et al.*, 2006), and Vps33p (Starai *et al.*, 2008). Moreover, Sec1p does not bind to the N-peptide or any other domain of its cognate syntaxin Sso1p (Togneri *et al.*, 2006), bolstering the argument that this binding mode is not required for general SM protein function. Instead, we favor the view that the essential role that SM proteins play in vesicle docking involves interactions with other regulatory proteins, such as Rabs and tethering complexes.

SM proteins have been functionally, and in some cases physically, linked to vesicle tethering complexes (Sato *et al.*, 2000; Seals *et al.*, 2000; Wiederkehr *et al.*, 2004; Wagner *et al.*, 2006; Peplowska *et al.*, 2007; Laufman *et al.*, 2009). Tethering precedes docking: tethering complexes reversibly attach vesicles to target membranes by using a mechanism intimately connected to the GTPase cycle of Rabs (Cai *et al.*, 2007). The yeast vacuolar SM protein Vps33p copurifies as a component of the homotypic vacuolar tethering complex HOPS (Sato *et al.*, 2000; Seals *et al.*, 2000), which interacts with the Rab Ypt7p (Mayer and Wickner, 1997; Ungermann *et al.*, 1998) to tether and dock vacuoles. The defect in class A *sec1* mutants is consistent with a block in secretion before SNARE complex assembly, possibly affecting the vesicle tethering step. Genetic studies also provide evidence for functional interaction between Sec1p and the exocyst tethering complex. A block in secretion caused by mutation or deletion of components of the exocyst tethering complex is suppressed by overexpression of Sec1p (Wiederkehr *et al.*, 2004). Furthermore, severe growth defects caused by combined lesions in exocyst component Sec3p (Finger and Novick, 2000; Roumanie *et al.*, 2005) and *sec1-1* (Novick and Schekman, 1979) suggest that Sec1p participates with or even in parallel to the exocyst to facilitate vesicle tethering and SNARE complex assembly. However, a robust physical interaction between Sec1p and tethering factors has been difficult to demonstrate (Wiederkehr *et al.*, 2004).

A link between SM proteins and tethering complexes indirectly connects SM protein function to Rab GTP activation of tethering; however, SM protein function may be more directly linked to Rabs. In chromaffin cells, Rab3A has been shown to interact with Munc18-1 at a loop region, near a peripheral helical cluster in domain 3b (Graham *et al.*, 2008). Similarly, yeast mutants *SLY1-20*[E532K], *SLY1-15*[T531I] and *SLY1-10*[ΔE532], which map to a short helical insertion in domain 3b, allow Sly1p to function independently of the yeast Rabs Ypt1p and Ypt6p (Dascher *et al.*, 1991; Li *et al.*, 2007). Strikingly, most of the class A *sec1* mutations map to the peripheral cluster of alpha helices in domain 3b, implicating this region for the essential function of Sec1p before vesicle docking and SNARE complex assembly. A Sec1p interaction with the Rab Sec4p could explain genetic evi-

dence for a functional interaction. The original *sec1-1*[G443E] allele, which also maps to domain 3b, is suppressed by multiple copies of *SEC4* (Salminen and Novick, 1987) and displays a severe growth defect in combination with loss-of-function mutation *sec4-8* (Finger and Novick, 2000). However, in contrast to the Rab3A findings, studies of Sec4p have not yet physically linked it to Sec1p.

After docking, SM proteins bind to SNARE complexes and stimulate membrane fusion (Scott *et al.*, 2004; Dulubova *et al.*, 2007; Shen *et al.*, 2007; Mima *et al.*, 2008), by using a mechanism that remains to be discovered. Although no SM protein-SNARE complex structure has been resolved, the syntaxin-binding cleft has been proposed as the likely binding site for the four-helix bundle of the SNARE complex (Sudhof and Rothman, 2009). Two separate mutations introduced at a Munc18-1/syntaxin 1a contact surface of domain 1, E59K and E66A, were reported to interfere with the interaction between Munc18-1 and the assembled synaptic SNARE complex (Deák *et al.*, 2009). We found that two simultaneous charge reversals at the analogous Munc18-1/syntaxin 1a contact site in domain 1 of Sec1p resulted in lethality (R63D, K64E; Table 2), although with no effect of alanine substitutions (*sec1-57*[R63A, K64A]), suggesting that an essential interaction with Sec1p is repelled by the acidic residues at this surface.

Surprisingly, isolation of class B mutants implicates another surface as critical for SNARE complex binding: a conserved groove opposite the syntaxin binding cleft. Alternatively, it is not the groove, per se, but a structural change induced by mutations in the groove that alter the SNARE complex-binding conformation. The conformation of SM proteins is expected to be altered by mutations at buried positions (Li *et al.*, 2005; Carpp *et al.*, 2006), a likely explanation for the defects we observe for the conserved site *sec1* mutants. However, we expect that charge alterations at the protein surface, such as those designed for groove mutant *sec1-58*, would disrupt a local interaction without disturbing the overall protein conformation. In support of this idea, Sec1p encoded by mutant *sec1-58* was expressed, purified and found to recapitulate the binding defect we observed in lysates. How the groove region is used to recognize and bind cognate SNARE complexes for activation of vesicle fusion awaits high-resolution structural analysis.

Assigning a regulatory role for yeast Sec1p in vesicle docking will require purification and reconstitution of exocytic docking intermediates, in vitro. It is likely that SM proteins are one component of a vesicle tethering/SNARE complex assembly/fusion activation machine, as has been demonstrated for Vps33p/HOPS in the highly coupled vesicle fusion reaction at the vacuole (Mima *et al.*, 2008; Starai *et al.*, 2008). Could SM protein-SNARE interactions form a scaffold around a fusion pore (Rizo *et al.*, 2006) and/or play a role in changing membrane curvature to resolve hemifusion, as has been proposed for the role of synaptotagmin and SNAREs in regulated exocytosis (Martens *et al.*, 2007)? Testing models for the mechanism of fusion activation will require high-resolution imaging of fusion intermediates, such as an SM protein-bound SNARE complex trapped between apposing membranes.

ACKNOWLEDGMENTS

We thank Edina Harsay for the generous gift of the Bgl2p antibody and for much advice, Mary Munson for the Sso1p antibody (Sso1-16,371), Wei Guo (University of Pennsylvania, Philadelphia, PA) for yeast strain *exo70-38*, Pat Brennwald (University of North Carolina School of Medicine, Chapel Hill, NC) for yeast strain *cdc42-6* and the Sec9p antibody (38B-3), and Peter Novick (University of California, San Diego, School of Medicine, La Jolla, CA) and Pat

Brennwald for the Sec1p antibody (Yu9). We also thank Barbara Siminovich-Blok and Rachel Schechter, for initial efforts in the random mutagenesis studies and John Togneri and Raj Patel for initial screening of *sec1* mutants. We are grateful to Pat Brennwald, Ruth Collins, Mary Munson, Vik Nanda, and Hays Rye for helpful discussions. This work was supported by the National Institutes of Health grant R01GM-066291 (to C.M.C.), the Pew Scholars Program for Biomedical Sciences (to C.M.C.), and National Science Foundation grant MCB 0447478 (to C. C.).

REFERENCES

- Adamo, J. E., Moskow, J. J., Gladfelter, A. S., Viterbo, D., Lew, D. J., and Brennwald, P. J. (2001). Yeast Cdc42 functions at a late step in exocytosis, specifically during polarized growth of the emerging bud. *J. Cell Biol.* **155**, 581–592.
- Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.
- Arac, D., Dulubova, I., Pei, J., Huryeva, I., Grishin, N. V., and Rizo, J. (2005). Three-dimensional structure of the rSly1 N-terminal domain reveals a conformational change induced by binding to syntaxin 5. *J. Mol. Biol.* **346**, 589–601.
- Aurora, R., and Rose, G. D. (1998). Helix capping. *Protein Sci.* **7**, 21–38.
- Bracher, A., Perrakis, A., Dresbach, T., Betz, H., and Weissenhorn, W. (2000). The X-ray crystal structure of neuronal Sec1 from squid sheds new light on the role of this protein in exocytosis. *Structure* **8**, 685–694.
- Bracher, A., and Weissenhorn, W. (2002). Structural basis for the Golgi membrane recruitment of Sly1p by Sed5p. *EMBO J.* **21**, 6114–6124.
- Brummer, M. H., Kivinen, K. J., Jantti, J., Toikkanen, J., Soderlund, H., and Keranen, K. (2001). Characterization of the *sec1-1* and *sec1-11* mutations. *Yeast* **18**, 1525–1536.
- Bryant, N. J., and James, D. E. (2001). Vps45p stabilizes the syntaxin homologue Tlg2p and positively regulates SNARE complex formation. *EMBO J.* **13**, 3380–3388.
- Burgoyne, R. D., Barclay, J. W., Ciuffo, L. F., Graham, M. E., Handley, M. T., and Morgan, A. (2009). The functions of Munc18-1 in regulated exocytosis. *Ann. N.Y. Acad. Sci.* **1152**, 76–86.
- Burkhardt, P., Hattendorf, D. A., Weis, W. I., and Fasshauer, D. (2008). Munc18a controls SNARE assembly through its interaction with the syntaxin N-peptide. *EMBO J.* **27**, 923–933.
- Cai, H., Reinisch, K., and Ferro-Novick, S. (2007). Coats, tethers, Rabs, and SNAREs work together to mediate the intracellular destination of a transport vesicle. *Dev. Cell* **12**, 671–682.
- Carpp, L. N., Ciuffo, L. F., Shanks, S. G., Boyd, A., and Bryant, N. J. (2006). The Sec1p/Munc18 protein Vps45p binds its cognate SNARE proteins via two distinct modes. *J. Cell Biol.* **173**, 927–936.
- Carr, C. M., Grote, E., Munson, M., Hughson, F. M., and Novick, P. J. (1999). Sec1p binds to SNARE complexes and concentrates at sites of secretion. *J. Cell Biol.* **146**, 333–344.
- Ciuffo, L. F., Barclay, J. W., Burgoyne, R. D., and Morgan, A. (2005). Munc18-1 regulates early and late stages of exocytosis via syntaxin-independent protein interactions. *Mol. Biol. Cell* **16**, 470–482.
- Dascher, C., Ossig, R., Gallwitz, D., and Schmitt, H. D. (1991). Identification and structure of four yeast genes (SLY) that are able to suppress the functional loss of YPT1, a member of the RAS superfamily. *Mol. Cell. Biol.* **11**, 872–885.
- Deák, F., Xu, Y., Chang, W.-P., Khvotchev, M., Dulubova, I., Liu, X., Sudhof, T. C., and Rizo, J. (2009). Munc18-1 binding to the neuronal SNARE complex controls synaptic vesicle priming. *J. Cell Biol.* **184**, 751–764.
- de Wit, H., Cornelisse, L. N., Toonen, R. F., and Verhage, M. (2006). Docking of secretory vesicles is syntaxin dependent. *PLoS ONE* **1**, e126.
- Dulubova, I., Khvotchev, M., Liu, S., Huryeva, I., Sudhof, T. C., and Rizo, J. (2007). Munc18-1 binds directly to the neuronal SNARE complex. *Proc. Natl. Acad. Sci. USA* **104**, 2697–2702.
- Dulubova, I., Yamaguchi, T., Gao, Y., Min, S. W., Huryeva, I., Sudhof, T. C., and Rizo, J. (2002). How Tlg2p/syntaxin 16 'snares' Vps45. *EMBO J.* **21**, 3620–3631.
- Finger, F. P., and Novick, P. (2000). Synthetic interactions of the post-Golgi sec mutations of *Saccharomyces cerevisiae*. *Genetics* **156**, 943–951.
- Gallwitz, D., and Jahn, R. (2003). The riddle of the Sec1/Munc-18 proteins—new twists added to their interactions with SNAREs. *Trends Biochem. Sci.* **28**, 113–116.
- Gietz, R. D., and Schiestl, R. H. (2007). Quick and easy yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.* **2**, 35–37.
- Goldstein, A., and Lampen, J. O. (1975). Beta-D-fructofuranoside fructohydrolase from yeast. *Methods Enzymol.* **42**, 504–511.
- Graham, M. E., Handley, M. T., Barclay, J. W., Ciuffo, L. F., Barrow, S. L., Morgan, A., and Burgoyne, R. D. (2008). A gain-of-function mutant of Munc18-1 stimulates secretory granule recruitment and exocytosis and reveals a direct interaction of Munc18-1 with Rab3. *Biochem. J.* **409**, 407–416.
- Grote, E., Carr, C. M., and Novick, P. J. (2000). Ordering the final events in yeast exocytosis. *J. Cell Biol.* **151**, 439–452.
- Guthrie C., and Fink G. R. (1991) Guide to Yeast Genetics and Molecular Biology. In: *Methods in Enzymology*, Vol. 94, ed. J. N. Ableson and M. I. Simon, San Diego, CA: Academic Press, 21–37.
- Hannenhalli, S. S., and Russell, R. B. (2000). Analysis and prediction of functional sub-types from protein sequence alignments. *J. Mol. Biol.* **303**, 61–76.
- Harsay, E., and Bretscher, A. (1995). Parallel secretory pathways to the cell surface in yeast. *J. Cell Biol.* **131**, 297–310.
- Harsay, E., and Schekman, R. (2007). Av19p, a member of a novel protein superfamily, functions in the late secretory pathway. *Mol. Biol. Cell* **18**, 1203–1219.
- He, B., Xi, F., Zhang, J., TerBush, D., Zhang, X., and Guo, W. (2007). Exo70p mediates the secretion of specific exocytic vesicles at early stages of the cell cycle for polarized cell growth. *J. Cell Biol.* **176**, 771–777.
- Hu, S. H., Latham, C. F., Gee, C. L., James, D. E., and Martin, J. L. (2007). Structure of the Munc18c/Syntaxin4 N-peptide complex defines universal features of the N-peptide binding mode of Sec1/Munc18 proteins. *Proc. Natl. Acad. Sci. USA* **104**, 8773–8778.
- Jahn, R. (2000). Sec1/Munc18 proteins: mediators of membrane fusion moving to center stage. *Neuron* **27**, 201–204.
- Johnson, J. M., Mason, K., Moallemi, C., Xi, H., Somaroo, S., and Huang, E. S. (2003). Protein family annotation in a multiple alignment viewer. *Bioinformatics* **19**, 544–545.
- Johnson, J. R., Ferdek, P., Lian, L. Y., Barclay, J. W., Burgoyne, R. D., and Morgan, A. (2009). Binding of UNC-18 to the N-terminus of syntaxin is essential for neurotransmission in *Caenorhabditis elegans*. *Biochem. J.* **418**, 73–80.
- Khvotchev, M., Dulubova, I., Sun, J., Dai, H., Rizo, J., and Sudhof, T. C. (2007). Dual modes of Munc18-1/SNARE interactions are coupled by functionally critical binding to syntaxin-1 N terminus. *J. Neurosci.* **27**, 12147–12155.
- Koumandou, V. L., Dacks, J. B., Coulson, R. M., and Field, M. C. (2007). Control systems for membrane fusion in the ancestral eukaryote; evolution of tethering complexes and SM proteins. *BMC Evol. Biol.* **7**, 29.
- Latham, C. F., *et al.* (2006). Molecular dissection of the Munc18c/syntaxin4 interaction: implications for regulation of membrane trafficking. *Traffic* **7**, 1408–1419.
- Laufman, O., Kedan, A., Hong, W., and Lev, S. (2009). Direct interaction between the COG complex and the SM protein, Sly1, is required for Golgi SNARE pairing. *EMBO J.* **28**, 2006–2017.
- Li, Y., Gallwitz, D., and Peng, R. (2005). Structure-based functional analysis reveals a role for the SM protein Sly1p in retrograde transport to the endoplasmic reticulum. *Mol. Biol. Cell* **16**, 3951–3962.
- Li, Y., Schmitt, H. D., Gallwitz, D., and Peng, R. W. (2007). Mutations of the SM protein Sly1 resulting in bypass of GTPase requirement in vesicular transport are confined to a short helical region. *FEBS Lett.* **581**, 5698–5702.
- Martens, S., Kozlov, M. M., and McMahon, H. T. (2007). How synaptotagmin promotes membrane fusion. *Science* **316**, 1205–1208.
- Mayer, A., and Wickner, W. (1997). Docking of yeast vacuoles is catalyzed by the Ras-like GTPase Ypt7p after symmetric priming by Sec18p (NSF). *J. Cell Biol.* **136**, 307–317.
- Mima, J., Hickey, C. M., Xu, H., Jun, Y., and Wickner, W. (2008). Reconstituted membrane fusion requires regulatory lipids, SNAREs and synergistic SNARE chaperones. *EMBO J.* **27**, 2031–2042.
- Misura, K. M., Scheller, R. H., and Weis, W. I. (2000). Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. *Nature* **404**, 355–362.
- Munson, M., and Bryant, N. J. (2009). A role for the syntaxin N-terminus. *Biochem. J.* **418**, e1–e3.
- Nicholson, K. L., Munson, M., Miller, R. B., Filip, T. J., Fairman, R., and Hughson, F. M. (1998). Regulation of SNARE complex assembly by an N-terminal domain of the t-SNARE Sso1p. *Nat. Struct. Biol.* **5**, 793–802.

- Novick, P., Field, C., and Schekman, R. (1980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell* 21, 205–215.
- Novick, P., and Schekman, R. (1979). Secretion and cell-surface growth are blocked in a temperature-sensitive mutant of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 76, 1858–1862.
- Peng, R., and Gallwitz, D. (2004). Multiple SNARE interactions of an SM protein: Sed5p/Sly1p binding is dispensable for transport. *EMBO J.* 23, 3939–3949.
- Peplowska, K., Markgraf, D. F., Ostrowicz, C. W., Bange, G., and Ungermann, C. (2007). The CORVET tethering complex interacts with the yeast Rab5 homolog Vps21 and is involved in endo-lysosomal biogenesis. *Dev. Cell* 12, 739–750.
- Rieder, S. E., and Emr, S. D. (1997). A novel RING finger protein complex essential for a late step in protein transport to the yeast vacuole. *Mol. Biol. Cell* 8, 2307–2327.
- Rizo, J., Chen, X., and Arac, D. (2006). Unraveling the mechanisms of synaptotagmin and SNARE function in neurotransmitter release. *Trends Cell Biol.* 16, 339–350.
- Roumanie, O., Wu, H., Molk, J. N., Rossi, G., Bloom, K., and Brennwald, P. (2005). Rho GTPase regulation of exocytosis in yeast is independent of GTP hydrolysis and polarization of the exocyst complex. *J. Cell Biol.* 170, 583–594.
- Salminen, A., and Novick, P. J. (1987). A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell* 49, 527–538.
- Sato, T. K., Rehling, P., Peterson, M. R., and Emr, S. D. (2000). Class C Vps protein complex regulates vacuolar SNARE pairing and is required for vesicle docking/fusion. *Mol. Cell* 6, 661–671.
- Schaffer, A. A., Aravind, L., Madden, T. L., Shavirin, S., Spouge, J. L., Wolf, Y. I., Koonin, E. V., and Altschul, S. F. (2001). Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. *Nucleic. Acids Res.* 29, 2994–3005.
- Schulze, K. L., Littleton, J. T., Salzberg, A., Halachmi, N., Stern, M., Lev, Z., and Bellen, H. J. (1994). *rop*, a *Drosophila* homolog of yeast Sec1 and vertebrate n-Sec1/Munc-18 proteins, is a negative regulator of neurotransmitter release in vivo. *Neuron* 13, 1099–1108.
- Scott, B. L., Van Komen, J. S., Irshad, H., Liu, S., Wilson, K. A., and McNew, J. A. (2004). Sec1p directly stimulates SNARE-mediated membrane fusion in vitro. *J. Cell Biol.* 167, 75–85.
- Seals, D. F., Eitzen, G., Margolis, N., Wickner, W. T., and Price, A. (2000). A Ypt/Rab effector complex containing the Sec1 homolog Vps33p is required for homotypic vacuole fusion. *Proc. Natl. Acad. Sci. USA* 97, 9402–9407.
- Shen, J., Tareste, D. C., Paumet, F., Rothman, J. E., and Melia, T. J. (2007). Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. *Cell* 128, 183–195.
- Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19–27.
- Starai, V. J., Hickey, C. M., and Wickner, W. (2008). HOPS proofreads the trans-SNARE complex for yeast vacuole fusion. *Mol. Biol. Cell* 19, 2500–2508.
- Sudhof, T. C., and Rothman, J. E. (2009). Membrane fusion: grappling with SNARE and SM proteins. *Science* 323, 474–477.
- Togneri, J., Cheng, Y. S., Munson, M., Hughson, F. M., and Carr, C. M. (2006). Specific SNARE complex binding mode of the Sec1/Munc-18 protein, Sec1p. *Proc. Natl. Acad. Sci. USA* 103, 17730–17735.
- Ungermann, C., Sato, K., and Wickner, W. (1998). Defining the functions of trans-SNARE pairs. *Nature* 396, 543–548.
- Voets, T., Toonen, R. F., Brian, E. C., de Wit, H., Moser, T., Rettig, J., Sudhof, T. C., Neher, E., and Verhage, M. (2001). Munc18-1 promotes large dense-core vesicle docking. *Neuron* 31, 581–591.
- Wagner, M. C., Molnar, E. E., Molitoris, B. A., and Goebel, M. G. (2006). Loss of the homotypic fusion and vacuole protein sorting or Golgi-associated retrograde protein vesicle tethering complexes results in gentamicin sensitivity in the yeast *Saccharomyces cerevisiae*. *Antimicrob. Agents Chemother.* 50, 587–595.
- Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H., and Rothman, J. E. (1998). SNAREpins: minimal machinery for membrane fusion. *Cell* 92, 759–772.
- Weimer, R. M., Richmond, J. E., Davis, W. S., Hadwiger, G., Nonet, M. L., and Jorgensen, E. M. (2003). Defects in synaptic vesicle docking in unc-18 mutants. *Nat. Neurosci.* 6, 1023–1030.
- Wiederkehr, A., De Craene, J. O., Ferro-Novick, S., and Novick, P. (2004). Functional specialization within a vesicle tethering complex: bypass of a subset of exocyst deletion mutants by Sec1p or Sec4p. *J. Cell Biol.* 167, 875–887.
- Wiederkehr, A., Du, Y., Pypaert, M., Ferro-Novick, S., and Novick, P. (2003). Sec3p is needed for the spatial regulation of secretion and for the inheritance of the cortical endoplasmic reticulum. *Mol. Biol. Cell* 14, 4770–4782.
- Yamaguchi, T., Dulubova, I., Min, S. W., Chen, X., Rizo, J., and Sudhof, T. C. (2002). Sly1 binds to Golgi and ER syntaxins via a conserved N-terminal peptide motif. *Dev. Cell* 2, 295–305.
- Zheng, L., Baumann, U., and Reymond, J. L. (2004). An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Res.* 32, e115.