Supporting Information

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SI Methods

Plasmids. The pYJ400 series plasmid vectors were generated by introducing the 720-bp upstream region and the 300-bp downstream region of the alcohol dehydrogenase *ADH1* gene into XhoI/HindIII and SacII/SacI sites of the pRS400 series vectors, as described (1). Genes encoding the neuronal SNARE proteins syntaxin-1, SNAP25, and synaptobrevin-2 were cloned by RT-PCR using mRNA prepared from PC-12 cells, digested with EcoRI and SacII, and inserted into the EcoRI and SacII sites of pYJ406, pYJ408, and pYJ406, respectively. The synaptobrevin mutants Syb2-VV, Syb2-GG, Syb2-EE, and Syb2-QQ were generated by PCR with specific primers, and the resulting PCR products were digested with EcoRI and SacII and inserted into the EcoRI and SacII sites of pYJ406. Munc18 was cloned from PC-12 cell mRNA by RT-PCR, digested with HindIII, and inserted into the HindIII site of the plasmid p405TDH3 (Addgene plasmid 15969).

Yeast Strains. *Saccharomyces cerevisiae* strains BJ3505 [*Mata ura3-52 trp1-Δ101 his3-Δ200 lys2-801 gal2* (*gal3*) *can1 prb1-Δ1.6R pep4::HIS3*] (2) and DKY6281 (*Matα ura3-52 leu2-3,112 trp1-Δ901 his3-Δ200 lys2-801 suc2-Δ9 pho8::TRP1*) (3), or their derivatives described below, were used to purify vacuoles for in vitro fusion assays. BJ3505 *nyv1Δ::TRP1 snc2Δ::Hygro*^{R} was transformed with BsaBI-linearized pYJ406-Syb2 to generate BJ3505 *nyv1Δ snc2Δ* expressing synaptobrevin-2 (BJ-*Syb2*). DKY6281 nyv1Δ::HIS3 was transformed with BsaBI-linearized pYJ406-Stx1 and pYJ408-S25 to generate DKY6281 *nyv1Δ* expressing both syntaxin-1 and SNAP-25 (DKY-*Stx1/S25*). The strain DKY-Stx1/S25 expressing Munc18 (DKY-*Stx1/S25/M18*) was generated by transforming the strain DKY-*Stx1/S25* with XcmI-linearized p405TDH3-Munc18. To generate BJ-*Syb2* containing vacuoles with luminal GFP (BJ-*Syb2*-GFP), BJ-*Syb2* was transformed with BsaBI-linearized pYJ400-mel-sGFP (4).

Reagents. Anti–syntaxin-1 (Santa Cruz), anti-VAMP2 (Abcam), anti-SNAP25 (Abcam), and anti–Munc18-1 (Santa Cruz) antibodies were purchased. Anti-Sec18p, anti-Vam3p, anti-Vps33p, and anti-Ypt7p antibodies were prepared as described (4) and dialyzed into PS buffer (10 mM Pipes/KOH at pH 6.8 and 200 mM sorbitol) with 125 mM KCl. The concentrations used (unless otherwise noted) were as follows: 0.54 μM affinity-purified anti-Sec18p antibody (5); 1.3 μM affinity-purified anti-Vam3p antibody; 0.48 μM affinity-purified anti-Vps33p antibody; and 11.5 μM affinity-purified anti-Ypt7p antibody. The cytosolic domain of synaptobrevin-2 (Syb2-CD) was produced as a his_6-fusion from *Escherichia coli* Rosetta 2 (λDE3) (Novagen) bearing pParallel-HIS-Syb2-CD. Full-length his_6-SNAP25 and his_6-Munc18 were produced from *E. coli* Rosetta 2 (λDE3) bearing pParallel-HIS-SNAP25 and pParallel-HIS-Munc18, respectively. The his_6-tagged botulinum neurotoxin serotype E light chain (BoNT/E LC) was produced from *E. coli* Rosetta 2 (λDE3) bearing pParallel-HIS-BoNT/E-LC. These proteins were prepared as described (6). His_6-Gyp1-46p and Gdi1p (GDI) were prepared as described (4, 7). MARCKS effector domain (MED) (8) was custom-synthesized by ANYGEN (Gwangju) and dissolved in PS buffer. Purified recombinant proteins were dialyzed into PS buffer with 125 mM KCl and used at the following concentrations: 5 μM his_6-Gyp1-46p, 3 μM GDI, 2 μM Syb2-CD, and 20 μM MED, unless otherwise noted.

Extraction and Measurement of Vacuolar Lipids. Vacuolar lipids were extracted by a modification of the Bligh–Dyer method (9), as described (10). Briefly, chloroform (100 μL) and methanol supplemented with 0.1 M HCl (200 μL) were added to 37 μg of vacuoles, as measured by protein content, in 80 μL of PS buffer, mixed thoroughly by vortexing, and incubated at room temperature (RT) for 1 h. After PS buffer (100 μL) and chloroform (100 μL) were added, the sample was vortexed thoroughly and centrifuged at 14,000 × *g* at RT for 30 s. The organic layer was transferred to a 13 × 100 mm bound-bottom glass tube. Chloroform (200 μL) was added to the remaining aqueous layer, the mixture was vortexed and centrifuged as above, and the organic layer was removed and added to the organic layer from the first extraction. PS buffer (360 μL) and methanol·HCl (400 μL) were added to the combined organic layers. This mixture was vortexed thoroughly and centrifuged at RT for 30 s, and the aqueous layer was removed by aspiration. The organic layer was then dried in a Sorvall Speed-Vac SC100 (Thermo Fisher Scientific) and used for estimation of vacuole lipid levels. Vacuole lipid levels were measured by using a lipid phosphorous assay. Ten microliters of 2% (wt/vol) ammonium molybdate was added to extracted vacuolar lipids and to standards (0, 5, 10, 25, 50, 75, 100, and 125 μL of a 1 mM NaH_2PO_4 solution). Samples were dried completely in Speed-Vac. After the addition of perchloric acid [300 μL of a 70% (vol/vol) solution], samples were incubated at 180 °C for 30 min, and then cooled to RT. Ammonium molybdate (1.5 mL of a 0.4% solution) and ascorbic acid [225 μL of a 10% (wt/vol) solution] were added. The mixture were incubated at 90 °C for 10 min, then and cooled to RT. Absorbance at 820 nm was measured, and phospholipid concentration was estimated by comparing vacuole lipid samples to NaH_2PO_4 standards. To obtain the lipid concentrations shown in Table S1, measured phospholipid concentrations were multiplied by 1.18 to correct for a reported ergosterol:phospholipid molar ratio of 0.18 in vacuolar lipids (11), as described (10).

Measurement of Vacuolar SNARE Levels. For estimation of vacuolar SNARE levels, BJ3505, DKY6281, BJ-*Syb2*, and DKY-*Stx1/S25* vacuoles were analyzed by SDS/PAGE and immunoblotting for Vam7p, Vam3p, Vti1p, Nyv1p, synaptobrevin-2, syntaxin-1, and SNAP25. Protein levels were estimated by comparing band intensities from vacuolar samples to band intensities from standards of purified recombinant Vam7p, Vam3p, Vti1p, Nyv1p (12), his_6-Syb2-CD, his_6-tagged syntaxin-1 cytosolic domain, and his_6-SNAP25.

1. Jun Y, Wickner W (2007) Assays of vacuole fusion resolve the stages of docking, lipid mixing, and content mixing. *Proc Natl Acad Sci USA* 104(32):13010–13015.
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5. Thorngren N, Collins KM, Fratti RA, Wickner W, Merz AJ (2004) A soluble SNARE drives rapid docking, bypassing ATP and Sec17/18p for vacuole fusion. *EMBO J* 23(14):2765–2776.
6. Jun Y, et al. (2006) Reversible, cooperative reactions of yeast vacuole docking. *EMBO J* 25(22):5260–5269.
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11. Zinser E, et al. (1991) Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae. *J Bacteriol* 173(6): 2026–2034.
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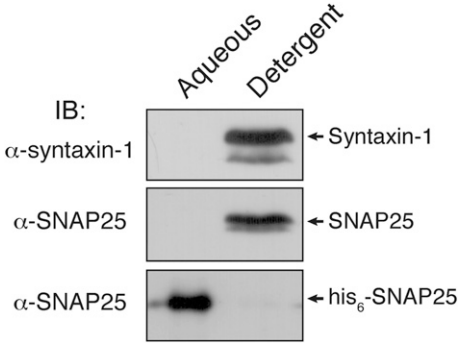

Fig. S1. Yeast-expressed vacuolar SNAP25 appears to be palmitoylated. Triton X-114 phase partitioning analysis of the hydrophobicity of yeast-expressed vacuolar SNAP25, his_6-SNAP25 expressed in *E. coli*, and yeast-expressed vacuolar syntaxin-1 with its transmembrane anchor was performed with DKY-*Stx1/S25* vacuoles supplemented with recombinant his_6-SNAP25, as described (1).

1. Bordier C (1981) Phase separation of integral membrane proteins in Triton X-114 solution. *J Biol Chem* 256(4):1604–1607.

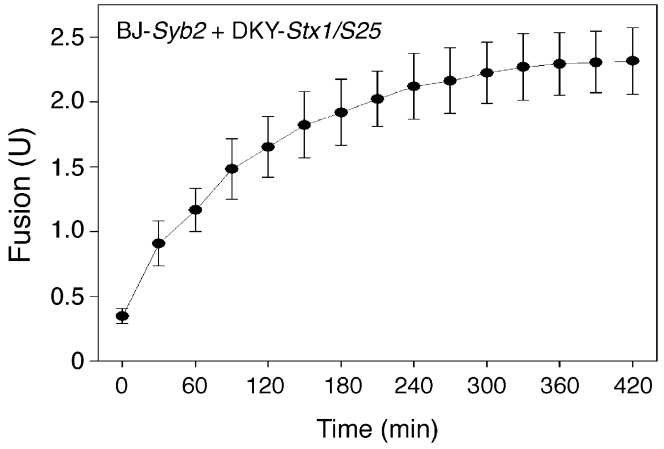

Fig. S2. Kinetics of neuronal SNARE-mediated vacuole fusion. BJ-*Syb2* vacuoles and DKY-*Stx1/S25* vacuoles were mixed and incubated in fusion reaction buffer (without ATP) at 27 °C. A portion of the reaction (30 μL) was transferred to ice at the times indicated. After 420 min, the activity of ALP was measured. The results are the mean of three independent experiments ± SEM.

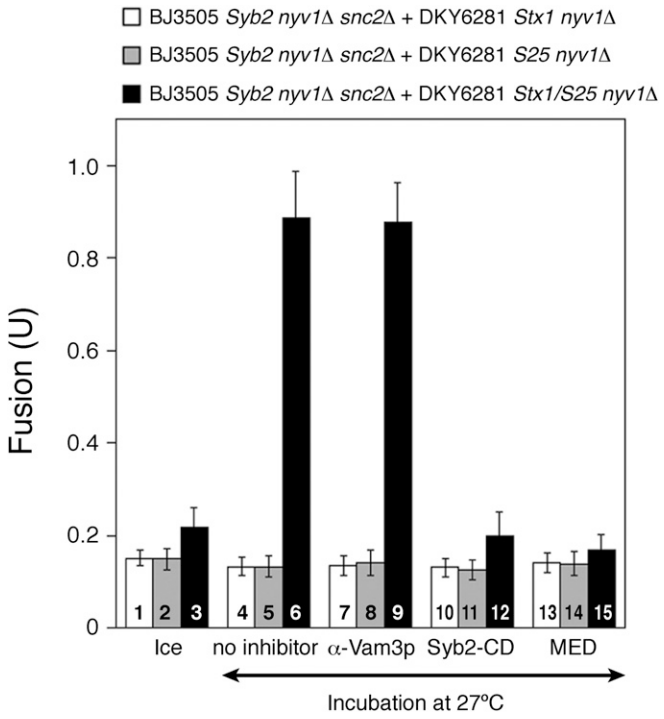

Fig. S3. Neuronal SNARE-driven vacuole fusion requires both syntaxin-1 and SNAP25. Vacuoles were purified from the yeast strains indicated and incubated in fusion reaction buffer at 27 °C. After 90 min, the activity of ALP was measured. The results are the mean of three independent experiments ± SEM.

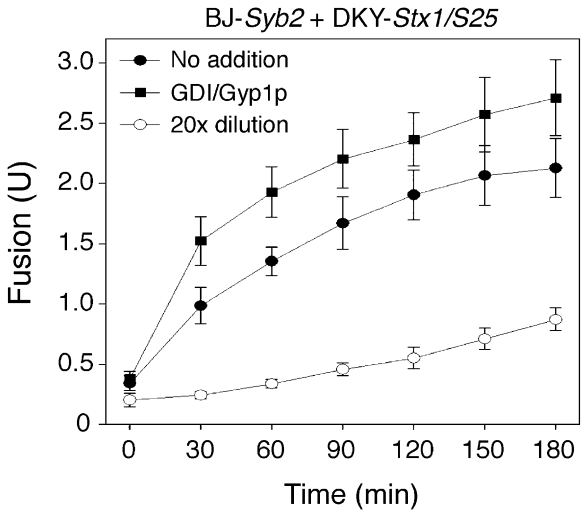

Fig. S4. Fusion kinetics of standard, GDI/Gyp1p-treated, and 20-fold diluted reactions. BJ-*Syb2* vacuoles and DKY-*Stx1/S25* vacuoles were mixed and incubated in the absence or presence of GDI/Gyp1p at 27 °C. For the 20× dilution, fusion reactions were diluted with reaction buffer and incubated at 27 °C. A portion of the reaction was transferred to ice at the times indicated. After 180 min, the activity of ALP was measured. The results are the mean of three independent experiments ± SEM.

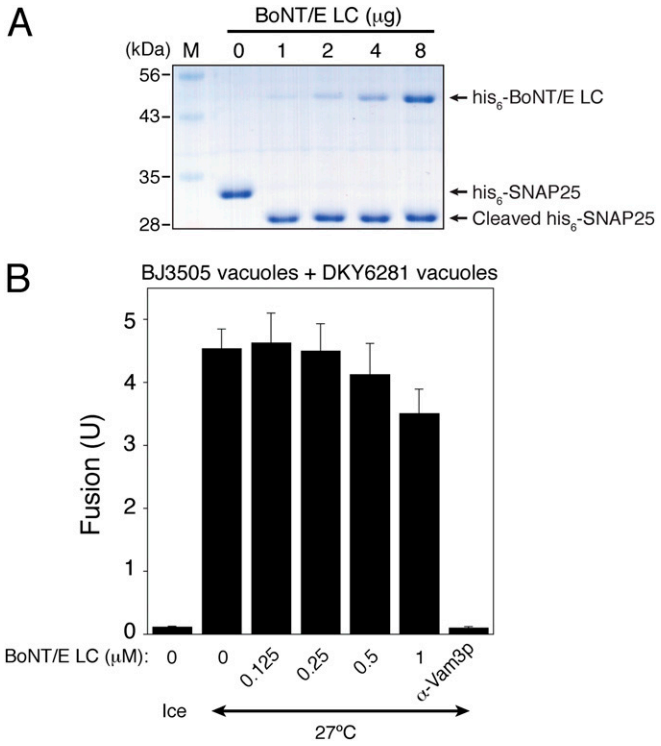

Fig. S5. Normal vacuole fusion is largely unaffected by BoNT/E light chain. (*A*) Recombinant his_6-SNAP25 was incubated with the indicated amounts of recombinant his_6-BoNT/E light chain at 27 °C. After 90 min, the proteins were resolved by SDS/PAGE and stained with Coomassie Brilliant Blue 250. (*B*) BJ3505 vacuoles (3 μg) and DKY6281 vacuoles (3 μg) were mixed and incubated at 27 °C with the indicated concentrations of BoNT/E LC. After 90 min, ALP activity was measured. The results are the mean of three independent experiments ± SEM.

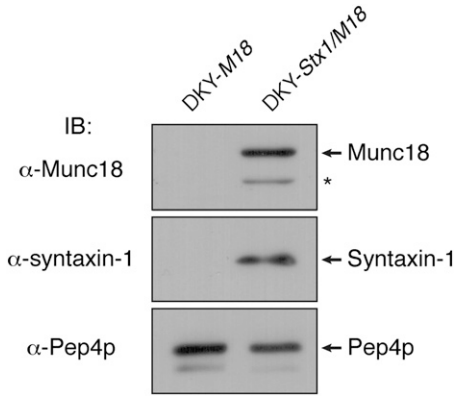

Fig. S6. Munc18 recruitment to the vacuole requires syntaxin-1. Immunoblotting analysis of purified vacuoles is shown. Vacuoles were isolated from the yeast strains indicated, and protein compositions were analyzed by immunoblotting. The asterisk indicates a degradation product of Munc18.

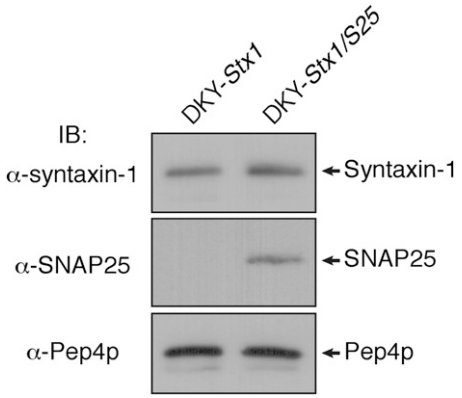

Fig. S7. The amount of vacuolar syntaxin-1 was comparable between DKY-*Stx1* and DKY-*Stx1/S25* vacuoles. Vacuoles were isolated from the DKY-*Stx1* and DKY-*Stx1/S25*, and protein compositions were analyzed by immunoblotting.

Table S1. Protein abundance, relative to lipids, in normal vacuoles or neuronal SNARE-expressing vacuoles

	Molar ratio of lipid:protein on vacuoles*			
Protein	BJ3505	DKY6281	BJ-*Syb2*	DKY-*Stx1/S25*
Vam3p	1.61×10^5	3.47×10^5	—	—
Vti1p	2.77×10^5	7.36×10^5	—	—
Vam7p	8.31×10^5	10.58×10^5	—	—
Nyv1p	11.8×10^5	13.6×10^5	—	—
Synaptobrevin-2	—	—	2.59×10^4	—
Syntaxin-1	—	—	—	4.96×10^4
SNAP25	—	—	—	16.0×10^4

*Based on measured (*SI Materials and Methods*) values of 2.08 nmol lipid per μg of total vacuole protein for BJ3505 vacuoles, 2.34 nmol lipid per μg of total vacuole protein for DKY6281 vacuoles, 2.23 nmol lipd per μg of total vacuole protein for BJ-*Syb2* vacuoles, and 2.35 nmol lipid per μg of total vacuole protein for DKY-*Stx1/S25* vacuoles.