Reconstituted membrane fusion requires regulatory lipids, SNAREs, and synergistic SNARE-chaperones

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Supplementary Materials and methods

Protein expression and purification

S. cerevisiae VAM3, VTI1, and NYV1 genes were subcloned into pGST-parallel1 vectors (Sheffield *et al*, 1999), encoding GST and a TEV protease sites. For Vam3p production, the linker region between TEV and VAM3 was deleted, to obtain full-length, untagged Vam3p with an extra N-terminal glycyl residue after cleavage by TEV protease. For Vti1p and Nyv1p, the GST and linker sequences upstream of VTI1 and NYV1 were deleted, yielding full-length untagged Vti1p and Nyv1p without extra residues.

SNAREs were produced in *E. coli* Rosetta2 (Novagen) in TB medium with ampicillin and chloramphenicol by induction with 1 mM IPTG (3h, 37ºC, 4 L). Cells were suspended in 40 ml HB (20 mM Hepes-NaOH, pH 8.0, 1 mM EDTA, 1 mM DTT) with 100 mM NaCl, lysed by French press, and centrifuged (60Ti [Beckman], 50,000 rpm, 30 min, 4ºC).

For GST-Vam3p, lysate pellets were resuspended in PBS with 1 mM EDTA, 1 mM DTT, 10% glycerol, and 100 mM β-OG and incubated (4ºC, 1 h) with gentle agitation. The lysate was centrifuged (60 Ti rotor, 50,000 rpm, 30 min, 4ºC) and the supernatant was applied to a 1.5 x 5 cm glutathione-Sepharose column in resuspension buffer. The column was washed with 100 ml of the resuspension buffer. GST-Vam3p was eluted with

100 mM Hepes-NaOH, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 mM β-OG, 10% glycerol, 20 mM reduced glutathione.

For Vti1p and Nyv1p, lysate pellets were re-suspended in 40 ml HB, 100 mM NaCl, 0.5% Thesit and incubated (4ºC, 1 h, gentle agitation). After centrifugation (60 Ti rotor, 50,000 rpm, 30 min, 4ºC), supernatants were loaded onto Q-Sepharose columns. After washing the columns with 100 ml of HB, 10% glycerol, 40 mM CHAPS, Vti1p and Nyv1p were eluted with HB, 500 mM NaCl, 10% glycerol, 40 mM CHAPS.

Full-length, untagged Vam7p was purified from *E. coli*, producing chitin-binding domain (CBD) -Vam7p fusion protein which was isolated, cleaved, and eluted as described (Starai *et al*, 2007). TEV protease (Jun *et al*, 2007), αVam3p (Collins *et al*, 2005), His_6-Sec17p (Haas and Wickner, 1996), His_6-Sec18p (Haas and Wickner, 1996), and HOPS complex bearing Vps33p-GST fusion protein (Starai *et al*, 2008) were purified as described. HOPS purified without a GST tag had the same activity in our lipid mixing assays.

For purification of Vps33p, *S. cerevisiae* strain CHY2 was constructed by transforming yeast FY834 (MATα, ura3-52, leu2Δ1, lys2Δ202, trp1Δ63, his3Δ200, GAL2+) (Winston *et al*, 1995) with linearized p403GAL1-VPS33-TEV-GST (Starai *et al*, 2008). CHY2 from solid CSM-his dropout medium (MP Biomedicals) was grown at 30°C in 8 L of YPD to $O.D._{600}$ ~ 2.0. Cells were harvested (Beckman JA10 rotor, 5000 rpm, 5 min, 23°C), resuspended in 500 ml Milli-Q water, centrifuged as above, resuspended in 30 ml of 20 mM Hepes-NaOH, pH 7.8, 200 mM NaCl, 2.5% glycerol, 5 mM 2-mercaptoethanol, PMSF (1 mM), leupeptin (0.46 μg/ml), pepstatin A (3.5 μg/ml), and pefabloc-SC (2.4 μg/ml) and frozen in liquid nitrogen. Frozen yeast cells were lysed in a Waring blender

(Goode *et al*, 1999), the powder was thawed, and the extract centrifuged (13,000 rpm, 20min, 4^{o}C, JA-20). Supernatants were collected, mixed with Triton X-100 (0.05 %), and incubated on ice for 20 minutes. Insoluble material was removed (60Ti, 30 min, 50,000 rpm, 4^{o}C) and the supernatant was passed through a 0.45 micron syringe driven filter (Millipore). The extract was applied to a 25x80 mm Glutathione Sepharose 4B (GE Healthcare) column in 20 mM Hepes-NaOH, pH 7.8, 200 mM NaCl, 5% glycerol, 5 mM 2-mercaptoethanol, 0.05 % Triton X-100 at 4^{o}C. The column was washed with 3 volumes of this buffer, then 3 volumes of 20 mM Hepes-NaOH, pH 7.8, 50 mM NaCl, 5% glycerol, 5 mM 2-mercaptoethanol, 0.05 % Triton X-100. Protein was eluted with this buffer containing 20 mM glutathione. Fractions containing Vps33 were pooled, mixed with TEV protease, and incubated at 4^{o}C overnight. The protein was then applied to a MonoQ 5/50 GL column (GE Healthcare), and eluted with a 20 ml linear gradient of 0.05 to 1.0M NaCl in 20 mM Hepes-NaOH, pH 7.8, 200 mM sorbitol, 10% glycerol, 5 mM 2-mercaptoethanol, 0.002 % Triton X-100.

Supplementary References

Goode BL, Wong JJ, Butty AC, Peter M, McCormack AL, Yates JR, Drubin DG, Barnes G (1999) Coronin promotes the rapid assembly and croos-linking of actin filaments and may link the actin and microtubule cytoskeletons in yeast. *J Cell Biol* **144:** 83-98

Sheffield P, Garrard S, Derewenda Z (1999) Overcoming expression and purification problems of RhoGDI using a family of “Parallel” expression vectors. *Protein Expres*

Purif **15:** 34-39

Winston F, Dollard C, Ricupero-Hovasse SL (1995) Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. *Yeast* **11:** 53-55

Supplementary Figure legends

Figure S1 Coomassie-stained gels of purified yeast vacuolar SNAREs, SNARE chaperones, and proteoliposomes bearing the SNAREs. (**A**) Purified SNAREs (GST-Vam3p, Vti1p, Vam7p, and Nyv1p) and SNARE chaperones (His_6-Sec17p, His_6-Sec18p, GST-HOPS, and Vps33p). (**B**-**D**) SNARE proteoliposomes with DO-lipids and lipid compositions of PC/PS (B), vacuolar lipids (C), and vacuolar lipids with DAG/PI(3)P/PI(4,5)P_2 (D). (**E**-**I**) SNARE proteoliposomes with PO-lipids and the complete lipid composition (vacuolar lipids with DAG/PI(3)P/PI(4,5)P_2) (E-I) or without DAG, PI(3)P/PI(4,5)P_2, ERG, PI(3)P, or PI(4,5)P_2 (H and I). Donor and acceptor liposomes are “D” and “A”, respectively. See Table SI and Materials and methods for the lipid and SNARE compositions of these liposomes.

Figure S2 Lipid mixing of vacuolar SNARE liposomes. Mean values and standard deviations from more than 3 independent experiments. (**A**) PC/PS liposome pairs bearing the 3Q-SNAREs and the R-SNARE. (**B**) PC/PS liposomes bearing all 4 SNAREs. (**C**) Vacuolar lipid (DO-lipid) liposome pairs bearing the 3Q-SNAREs and the R-SNARE. (**D**) Vacuolar lipid (DO-lipid) liposomes bearing all 4 SNAREs. (**E**) Vacuolar (DO-lipid)

and regulatory lipids liposomes bearing all 4 SNAREs.

Figure S3 Lipid mixing of vacuolar SNARE liopsomes with Sec17p/Sec18p and HOPS. (**A**) HOPS-dependent enhancement of lipid mixing of vacuolar lipid (DO-lipid) liposome pairs bearing the 3Q-SNAREs and the R-SNARE, in the presence of Sec17p and Sec18p. (**B**) Lipid mixing of vacuolar lipid (DO-lipid) liposomes bearing all 4 SNAREs requires Sec17p/Sec18p. (**C**) Lipid mixing of vacuolar lipid (DO-lipid) liposomes bearing all 4 SNAREs requires HOPS. Lipid mixing assays were performed as described in Materials and methods.

Figure S1. Mima et al.

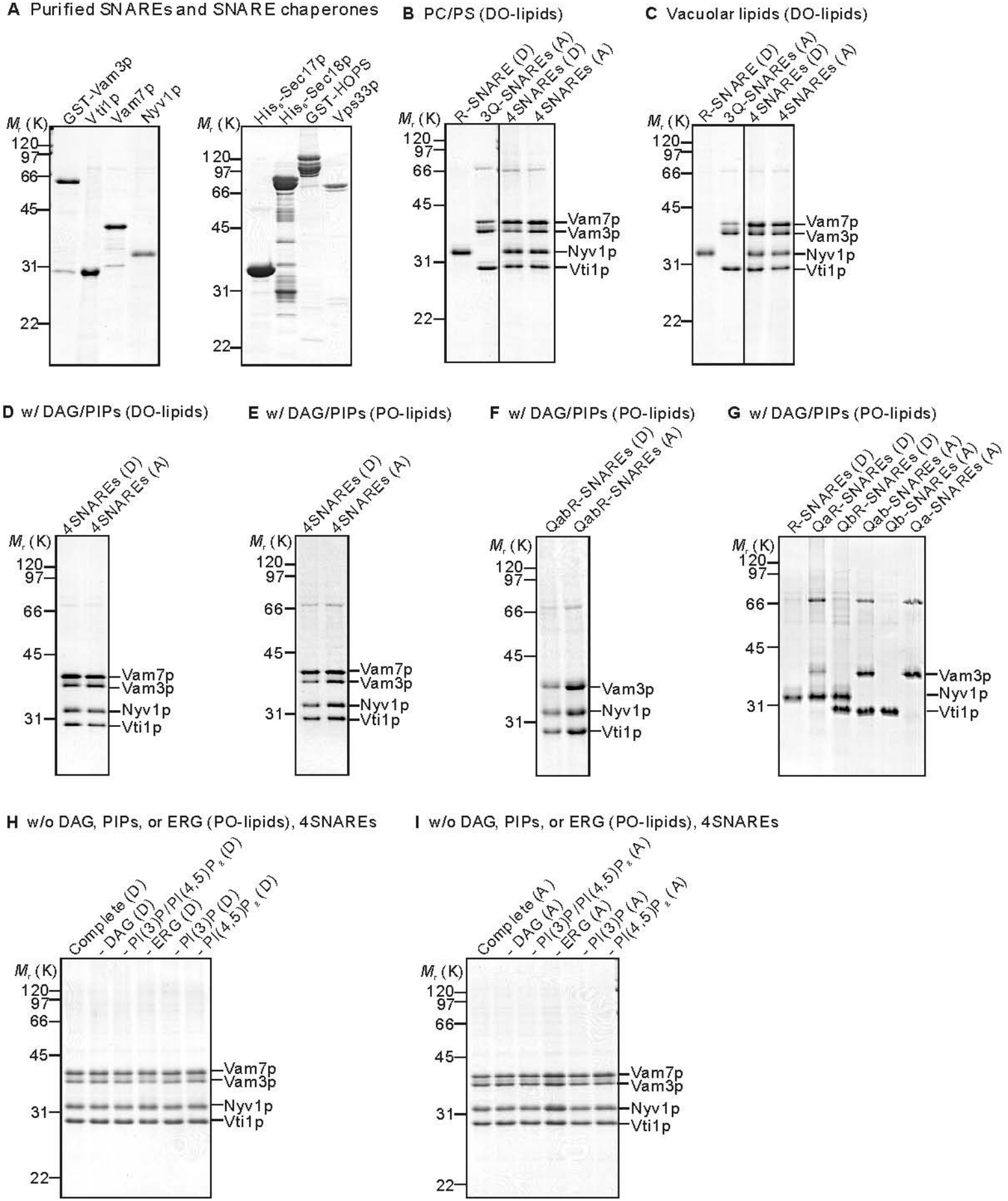


Figure S2. Mima et al.

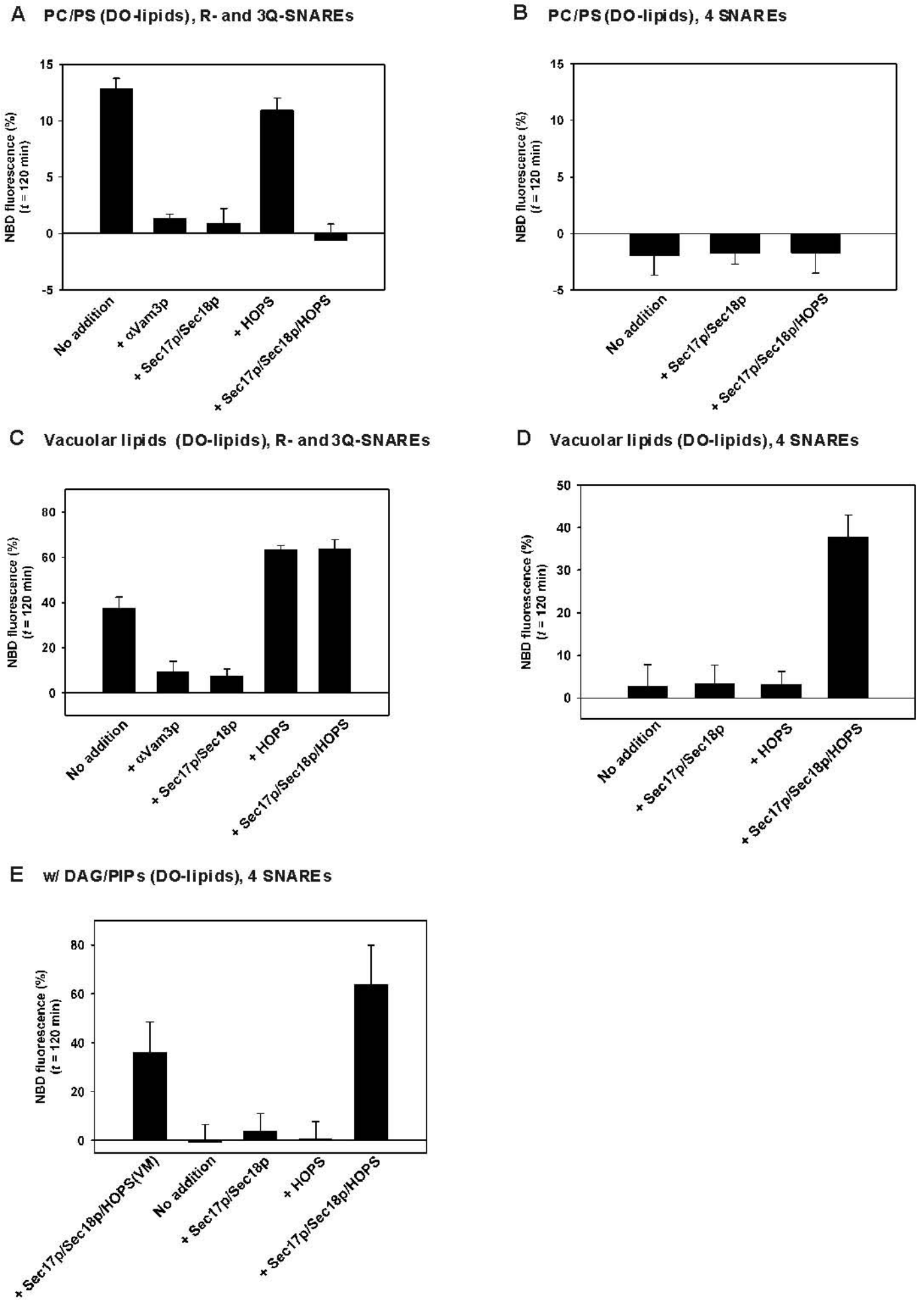
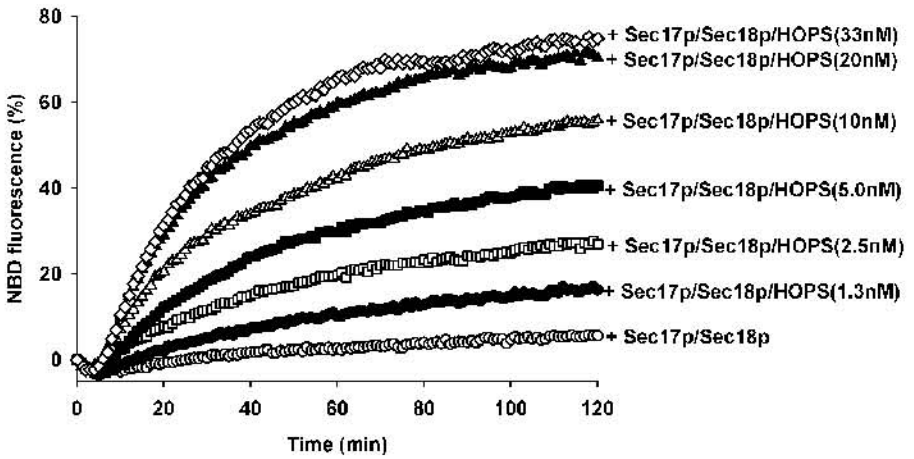
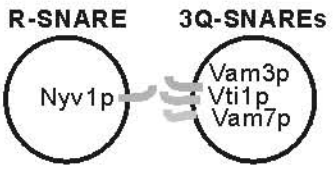


Figure S3. Mima et al.

A

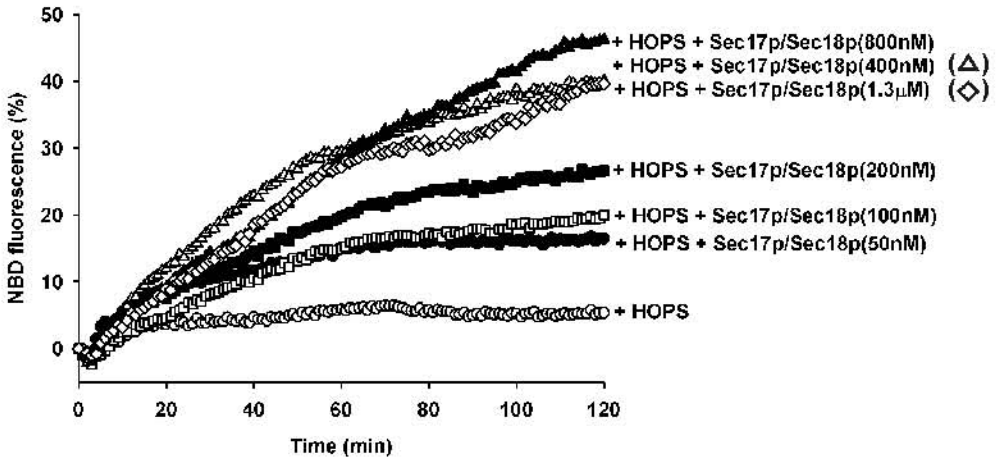


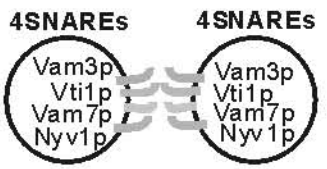


Lipids
DOPC
DOPE
soyPI
DOPS
DOPA
CL
ERG

Chaperones
Sec17p (400 nM)
Sec18p (400 nM)
HOPS (1.3 - 33 nM)

B

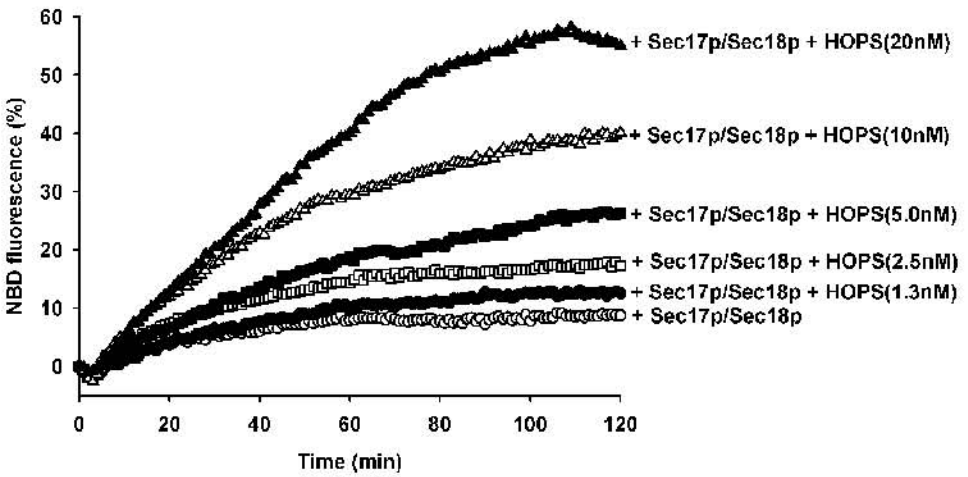


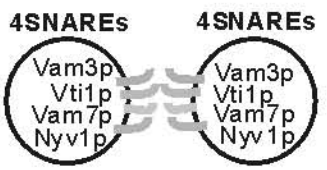


Lipids
DOPC
DOPE
soyPI
DOPS
DOPA
CL
ERG

Chaperones
Sec17p (50 nM - 1.3 μM)
Sec18p (50 nM - 1.3 μM)
HOPS (10 nM)

C





Lipids
DOPC
DOPE
soyPI
DOPS
DOPA
CL
ERG

Chaperones
Sec17p (400 nM)
Sec18p (400 nM)
HOPS (1.3 - 20 nM)

Table SI SNARE densities of reconstituted proteoliposomes bearing yeast vacuolar SNAREs used in this study

RPLs	Lipids[1]	SNAREs	Lipid/SNARE[2] (mole/mole)	Figures
R-SNARE, donor	PC/PS (DO)	Nyv1p	530	1A
3Q-SNAREs, acceptor	PC/PS (DO)	Vam3p	1000	1A
		Vti1p	710	
		Vam7p	1500	
4SNAREs, donor	PC/PS (DO)	Vam3p	1300	1B
		Vti1p	770	
		Vam7p	910	
		Nyv1p	630	
4SNAREs, acceptor	PC/PS (DO)	Vam3p	910	1B
		Vti1p	710	
		Vam7p	770	
		Nyv1p	650	
R-SNARE, donor	Vacuole (DO)	Nyv1p	590	2A
3Q-SNAREs, acceptor	Vacuole (DO)	Vam3p	800	2A
		Vti1p	690	
		Vam7p	1800	
4SNAREs, donor	Vacuole (DO)	Vam3p	1100	2B
		Vti1p	740	
		Vam7p	740	
		Nyv1p	710	
4SNAREs, acceptor	Vacuole (DO)	Vam3p	1200	2B
		Vti1p	830	
		Vam7p	830	
		Nyv1p	710	
4SNAREs, donor	Vacuole/DAG/PIPs (DO)	Vam3p	1100	2C
		Vti1p	830	
		Vam7p	690	
		Nyv1p	910	
4SNAREs, acceptor	Vacuole/DAG/PIPs (DO)	Vam3p	1000	2C
		Vti1p	950	
		Vam7p	830	
		Nyv1p	1100	
4SNAREs, donor	Vacuole/DAG/PIPs (PO)	Vam3p	2400	2D, 3
		Vti1p	1100	
		Vam7p	1300	
		Nyv1p	1400	
4SNAREs, acceptor	Vacuole/DAG/PIPs (PO)	Vam3p	1400	2D, 3
		Vti1p	1000	
		Vam7p	1100	
		Nyv1p	1100	

4SNAREs, donor	Vacuole/DAG/PIPs (PO) NBD-PS diI(5)C18ds	Vam3p Vti1p Vam7p Nyv1p	1300 770 670 870	3E
QabR-SNAREs, donor	Vacuole/DAG/PIPs (PO)	Vam3p Vti1p Nyv1p	1500 1100 1100	5A
QabR-SNAREs, acceptor	Vacuole/DAG/PIPs (PO)	Vam3p Vti1p Nyv1p	450 480 500	5A
R-SNARE, donor	Vacuole/DAG/PIPs (PO)	Nyv1p	710	5B, E
Qab-SNAREs, acceptor	Vacuole/DAG/PIPs (PO)	Vam3p Vti1p	690 500	5B, E
QaR-SNAREs, donor	Vacuole/DAG/PIPs (PO)	Vam3p Nyv1p	2000 490	5C
Qb-SNAREs, acceptor	Vacuole/DAG/PIPs (PO)	Vti1p	490	5C
QbR-SNAREs, donor	Vacuole/DAG/PIPs (PO)	Vtip Nyv1p	480 420	5D
Qa-SNAREs, acceptor	Vacuole/DAG/PIPs (PO)	Vam3p	570	5D
4SNAREs, donor	Complete (Vacuole/DAG/PIPs) (PO)	Vam3p Vti1p Vam7p Nyv1p	1500 740 1100 830	6
4SNAREs, acceptor	Complete (PO)	Vam3p Vti1p Vam7p Nyv1p	1400 770 1200 910	6
4SNAREs, donor	- DAG (PO)	Vam3p Vti1p Vam7p Nyv1p	1400 710 1100 910	6
4SNAREs, acceptor	- DAG (PO)	Vam3p Vti1p Vam7p Nyv1p	1200 650 1100 830	6
4SNAREs, donor	- ERG (PO)	Vam3p Vti1p Vam7p Nyv1p	2300 800 1200 830	6

4SNAREs, acceptor	- ERG (PO)	Vam3p	710	6
		Vti1p	490	
		Vam7p	610	
		Nyv1p	470	
4SNAREs, donor	- PI(3)P (PO)	Vam3p	1700	6
		Vti1p	690	
		Vam7p	1000	
		Nyv1p	910	
4SNAREs, acceptor	- PI(3)P (PO)	Vam3p	1300	6
		Vti1p	740	
		Vam7p	1100	
		Nyv1p	910	
4SNAREs, donor	- PI(4,5)P_2 (PO)	Vam3p	1500	6
		Vti1p	710	
		Vam7p	950	
		Nyv1p	770	
4SNAREs, acceptor	- PI(4,5)P_2 (PO)	Vam3p	1300	6
		Vti1p	650	
		Vam7p	950	
		Nyv1p	870	

[1]See Materials and methods for the details of the lipid compositions.

[2]See Materials and methods for determination of lipid and SNARE concentrations of the proteoliposomes.