**Table I.** Strains and plasmids used in this study

| Name | Genotype | Reference/source |
|---|---|---|
| *S. cerevisiae* | | |
| SEY6210 | *MATa leu2-3,112 ura3-52 his3-200 trp1-901 lys2-801 suc2-9* | (Robinson *et al.*, 1988) |
| BHY10 | SEY6210 *CPY-Invertase::LEU2* (pBHY11) | (Horazdovsky *et al.*, 1994) |
| BY4742 | *MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* | ATCC |
| BY4742 *vps11*Δ | BY4742; *vps11*Δ::KAN | Invitrogen |
| BY4742 *vps16*Δ | BY4742; *vps16*Δ::KAN | Invitrogen |
| BY4742 *vps18*Δ | BY4742; *vps18*Δ::KAN | Invitrogen |
| AMY1275 | BY4742; *vps33*Δ::KAN | This work |
| BY4742 *vps39*Δ | BY4742; *vps39*Δ::KAN | Invitrogen |
| BY4742 *vps41*Δ | BY4742; *vps41*Δ::KAN | Invitrogen |
| BLY1 | BHY10 *vps33*Δ*::KAN* | This work |
| BY4742 *pep4*Δ | BY4742 *pep4*Δ*::neo* | (Collins *et al*, 2007) |
| BLY2 | BY4742 *pep4*Δ*::neo vps33*Δ*::KAN* | This work |
| BLY3 | BHY10 *VPS33-ttx-GFP*$_{A207K}$*::NAT* | This work |
| BLY4 | BHY10 *vps33*$^{D88K}$*-ttx-GFP*$_{A207K}$*::NAT* | This work |
| BLY5 | BHY10 *vps33*$^{R281A}$*-ttx-GFP*$_{A207K}$*::NAT* | This work |
| BLY6 | BHY10 *vps33*$^{G297V}$*-ttx-GFP*$_{A207K}$*::NAT* | This work |
| BLY7 | BHY10 *vps33*$^{D300G}$*-ttx-GFP*$_{A207K}$*::NAT* | This work |
| *Yeast Expression Plasmids* | | |
| pRS416 | *URA3 CEN/ARSH4 Amp*$^{R}$ | (Sikorski & Hieter, 1989) |
| pBL1 | *pRS416 VPS33pr::VPS33-ttx-GFP*$_{A207K}$ | This work |
| pBL2 | *pRS416 VPS33pr::vps33*$^{L75P}$*-ttx-GFP*$_{A207K}$ | This work |
| pBL3 | *pRS416 VPS33pr::vps33*$^{D88K}$*-ttx-GFP*$_{A207K}$ | This work |
| pBL4 | *pRS416 VPS33pr::vps33*$^{I278N}$*-ttx-GFP*$_{A207K}$ | This work |
| pBL5 | *pRS416 VPS33pr::vps33*$^{R281A}$*-ttx-GFP*$_{A207K}$ | This work |
| pBL6 | *pRS416 VPS33pr::vps33*$^{G297V}$*-ttx-GFP*$_{A207K}$ | This work |
| pBL7 | *pRS416 VPS33pr::vps33*$^{D300G}$*-ttx-GFP*$_{A207K}$ | This work |
| pBL8 | *pRS416 VPS33pr::vps33*$^{D300E}$*-ttx-GFP*$_{A207K}$ | This work |

| | | |
|---|---|---|
| pBL9 | *pRS416 VPS33pr::vps33$^{F305L}$-ttx-GFP$_{A207K}$* | This work |
| pBL10 | *pRS416 VPS33pr::vps33$^{T553I}$-ttx-GFP$_{A207K}$* | This work |
| pBL11 | *pRS416 VPS33pr::vps33$^{E653A}$-ttx-GFP$_{A207K}$* | This work |

### *Bacterial Expression Plasmids*

| | | |
|---|---|---|
| pHIS parallel1 | pET22B- *amp$^R$ GST-(tev)-* | (Sheffield *et al.*, 1999) |
| pGST parallel1 | pGEX4T1- *amp$^R$ GST-(tev)-* | (Sheffield *et al.*, 1999) |
| pRP1 | pRSF- *kan$^R$ His$_7$-MBP-(tev)-* | This work |
| pBL12 | pRSF- *kan$^R$ His6-GFP$_{A207K}$-(tev)-* | This work |
| pBL12 | pRSF- *kan$^R$ -(tev)-GST* | This work |
| pBL13 | pBL12- *kan$^R$ SSO1(1-265)-(tev)-GST* | This work |
| pBL14 | pBL12- *kan$^R$ VAM3 (1-264)-(tev)-GST* | This work |
| pBL15 | pBL12- *kan$^R$ VAM3$_{Habc}$ (1-145)-(tev)-GST* | This work |
| pBL16 | pBL12- *kan$^R$ VAM3$_{Linker}$ (116-186)-(tev)-GST* | This work |
| pBL17 | pBL12- *kan$^R$ VAM3$_{SNARE}$ (182-264)-(tev)-GST* | This work |
| - | pGST parallel1- *amr$^R$* GST-Vti1 (1-194) | (Stroupe *et al.*, 2006) |
| AMB73 | pGEX-KET- *amp$^R$ GST-VAM7 (2-316)* | (Merz *et al.*, 2004) |
| AMB74 | pGEX-KET- *amp$^R$ GST-VAM7$_{PX}$ (2-123)* | (Merz *et al.*, 2004) |
| pBL18 | pGST paralle1- *amp$^R$ GST-(tev)-NYV1 (1-231)* | This work |
| pBL19 | pRP1- *kan$^R$ His$_7$-MBP-(tev)-VTI1 (1-194)* | This work |
| AMB225 | pTYB3- *amp$^R$ His6-Vam7 (1-316)* | (Schwartz *et al.*, 2009) |
| pBL20 | pHIS paralle1- *amp$^R$ His$_6$-(tev)-NYV1 (1-231)* | This work |
| pBL21 | pHIS paralle1- *amp$^R$ His$_6$-(tev)-NYV1$_{5\Delta}$ (1-211)* | This work |
| pBL22 | pBL12- *kan$^R$ His6-GFP$_{A207K}$-(tev)-Vam7 (190-316)* | This work |
| pBL23 | pGST paralle1- *amp$^R$ GST-(tev)-Vam7$_{SNARE}$ (190-316)* | This work |
| pBL24 | pGST paralle1- *amp$^R$ GST-(tev)-NYV1$_{SNARE}$ (162-231)* | This work |
| pBL25 | pGST paralle1- *amp$^R$ GST-(tev)-SED5$_{SNARE}$ (170-319)* | This work |
| pBL26 | pHIS paralle1- *amp$^R$ His$_6$-(tev)-BOS1 (1-222)* | This work |

| | | |
|---|---|---|
| pBL27 | pHIS paralle1- *amp$^R$ His$_6$-(tev)-SE22 (1-188)* | This work |
| pSN358 | pET14b- *amp$^R$ His$_6$-BET1 (1-123)* | (Stone *et al.,* 1997) |
| pBL28 | pGST paralle1- *amp$^R$ GST-(tev)-YKT6 (1-195)* | This work |
| pBL29 | pGST paralle1- *amp$^R$ GST-(tev)-SE22 (1-188)* | This work |
| pBL30 | pHIS paralle1- *amp$^R$ His$_6$-(tev)-YKT6 (1-195)* | This work |
| pBL31 | pBL17- *kan$^R$ VAM3$_{SNARE(-9\Delta)}$ (192-264)-(tev)-GST* | This work |
| pBL32 | pBL17- *kan$^R$ VAM3$_{SNARE(-7\Delta)}$ (199-264)-(tev)-GST* | This work |
| pBL33 | pBL17- *kan$^R$ VAM3$_{SNARE(-5\Delta)}$ (206-264)-(tev)-GST* | This work |
| pBL34 | pBL17- *kan$^R$ VAM3$_{SNARE(-3\Delta)}$ (213-264)-(tev)-GST* | This work |
| pBL35 | pBL17- *kan$^R$ VAM3$_{SNARE(-1\Delta)}$ (220-264)-(tev)-GST* | This work |
| pBL36 | pBL17- *kan$^R$ VAM3$_{SNARE(+1\Delta)}$ (227-264)-(tev)-GST* | This work |
| pBL37 | pBL17- *kan$^R$ VAM3$_{SNARE(+3\Delta)}$ (234-264)-(tev)-GST* | This work |
| pBL38 | pBL17- *kan$^R$ VAM3$_{SNARE(+5\Delta)}$ (241-264)-(tev)-GST* | This work |
| pBL39 | pBL17- *kan$^R$ SSO1$_{SNARE}$ (184-265)-(tev)-GST* | This work |
| pBL40 | pGST paralle1- *amp$^R$ GST-(tev)- VAM3$_{SNARE}$ (182-264)* | This work |
| pBL41 | pBL40- *amp$^R$ GST-(tev)- VAM3$_{SNARE(+9\Delta)}$ (182-257)* | This work |
| pBL42 | pBL40- *amp$^R$ GST-(tev)- VAM3$_{SNARE(+7\Delta)}$ (182-250)* | This work |
| pBL43 | pBL40- *amp$^R$ GST-(tev)- VAM3$_{SNARE(+5\Delta)}$ (182-243)* | This work |
| pBL44 | pBL40- *amp$^R$ GST-(tev)- VAM3$_{SNARE(+3\Delta)}$ (182-236)* | This work |
| pBL43 | pBL40- *amp$^R$ GST-(tev)- VAM3$_{SNARE(-1\Delta)}$ (182-222)* | This work |
| pBL43 | pBL40- *amp$^R$ GST-(tev)- VAM3$_{SNARE(-5\Delta)}$ (182-208)* | This work |
| pBL44 | pBL18- *amp$^R$ GST-(tev)-NYV1$_{(+9\Delta)}$ (1-225)* | This work |
| pBL45 | pBL18- *amp$^R$ GST-(tev)-NYV1$_{+7\Delta)}$ (1-218)* | This work |
| pBL46 | pBL18- *amp$^R$ GST-(tev)-NYV1$_{(+5\Delta)}$ (1-211)* | This work |
| pBL47 | pBL18- *amp$^R$ GST-(tev)-NYV1$_{(+5\Delta)}$ (1-211)* | This work |
| pBL47 | pBL12- *kan$^R$ PEP12 (1-268)-(tev)-GST* | This work |
| pBL48 | pBL12- *kan$^R$ SED5 (1-319)-(tev)-GST* | This work |

## SUPPLEMENTAL FIGURE LEGENDS

**Figure S1.** Vps11 and Vps18 required for interaction between HOPS and the Vam3-$H_{abc}$. As in Figure 1B, the cytoplasmic domain of Vam3 (1-264), $H_{abc}$ domain (1-145), linker domain (116-186), and SNARE domain (182-264) were expressed as C-terminal GST fusions. GST and the exoctyic Qa SNARE, Sso1-GST (1-265), were negative controls. Coomassie stained gel of the of GST-fusions proteins shown. ~450 $OD_{600\ nm}$ × mL of yeast detergent lysate were incubated with resins for 2 h at 4˚ C, washed three times with binding buffer, eluted, and samples were analyzed by SDS-PAGE and western blot (WB) for Vps11 or Vps18 and Vps33.

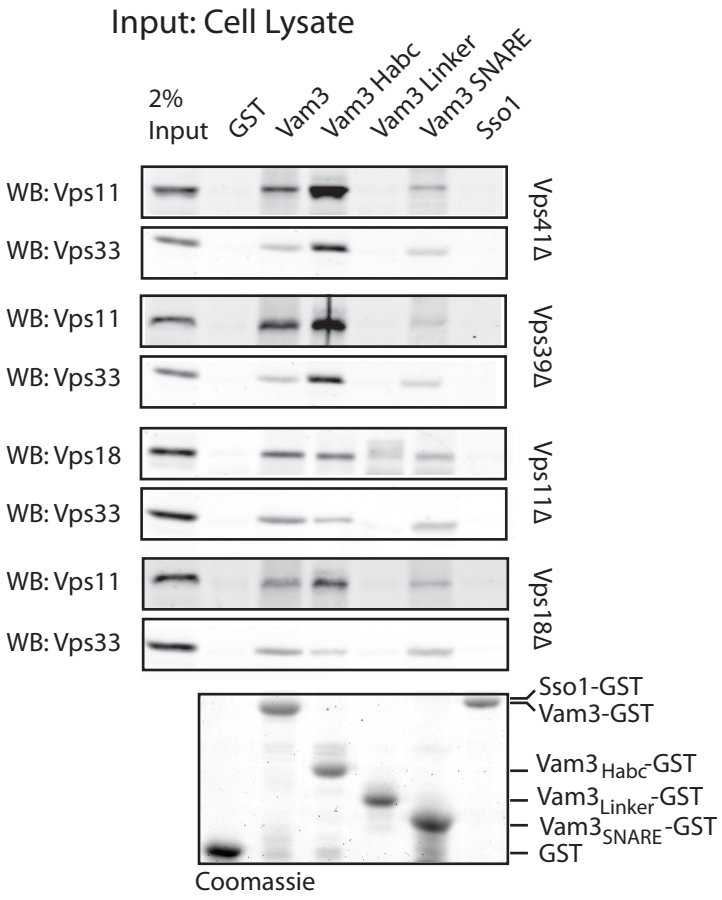
**Figure S2.** Vps33 binds vacuole Qa, Qc, and R SNAREs and the quaternary SNARE complex. As in Figure 2A, the C-terminal GST fusions were made to the cytoplasmic domains of Vam3, Vti1, Vam7, and Nyv1. SNARE complexes were formed of cytoplasmic domain of Vam3. Sso1 was used as a negative control. ~30 µg (1 µM) of each GST-fusion protein were incubated with 10.5 µg (~0.25 µM) purified Vps33 for 2 h at 30˚ C, washed three times with binding buffer, and eluted with binding buffer supplemented with 10mM reduced glutathione, pH 7.4. Samples were analyzed by SDS-PAGE and western blot for Vps33.

**Figure S3.** Vps33 binds Pep12. (A) For pulldowns on Vam3 or Pep12, the cytoplasmic domain of Vam3 (1-264) or Pep12 (1-268) were expressed as C-terminal GST fusions. GST and the exoctyic Qa SNARE, Sso1-GST (1-265), were negative controls. Coomassie stained gel of the of GST-fusions proteins shown. ~450 $OD_{600\ nm}$ × mL of yeast detergent lysate were incubated with resins for 2 h at 4˚ C, washed three times with binding buffer, eluted, and samples were analyzed by SDS-PAGE and western blot (WB) for Vps41, Vps11 and Vps33. (B) The cytoplasmic domains of the Vam3 and Pep12 were fused to GST. C-terminal GST-fusions of Sed5 (1-319), Sso1 (1-265), and GST were used as negative controls. 1 µg of purified Vps33 was incubated resins pre-bound to GST-fusion proteins for 2 h at 4˚ C. Resins were washed, and bound fractions were eluted and analyzed by SDS-PAGE and western blot for Vps33.

**Figure S4.** Vps33 binds Vam7 through its SNARE domain. GST-fusions to the Vam7 cytoplasmic domain, $Vam7_{PX}$, $Vam7_{SNARE}$, and the vacuole SNARE complex were used to pulldown purified Vps33. Sec22 and GST were used as negative controls. SNARE complexes were formed using purified Vam3-GST (1-264) bound to GSH resin and incubated overnight at 4˚ C with a five-fold excess of purified $His_7$-MBP-Vti1 (1-194), $His_6$-GFP-$Vam7_{SNARE}$ (190-316), and $His_6$-Nyv1 (1-231). 10.5 µg of purified Vps33 was incubated for 2 h at 30˚ C, washed three times with binding buffer, and eluted. Samples were separated by SDS-PAGE and analyzed by western blot for Vps33.

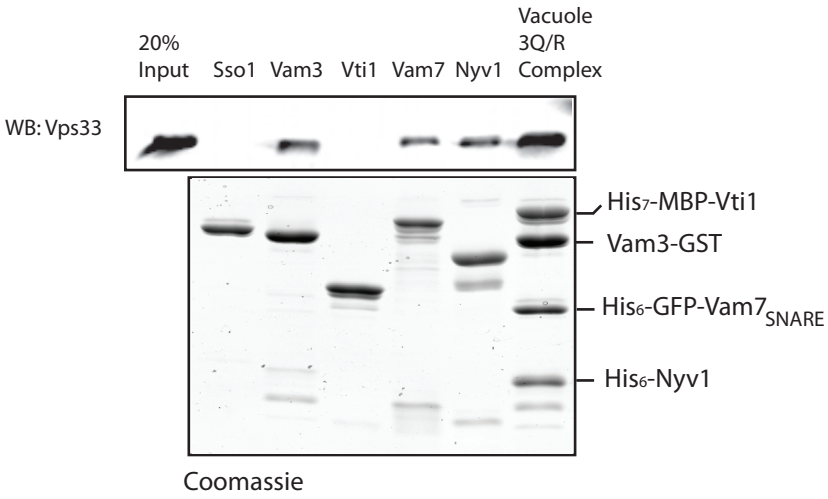
**Figure S5.** Characterization of chromosomal integrations of Vps33 point mutations. (A) Cultures grown overnight at 24˚ C were plated onto SC-URA, YPD, or YPD+5mM $ZnCl_2$ as 20-fold dilutions from 1.0 $OD_{600\ nm}$ × mL. Plates were incubated for 48 h and imaged. (B) Strains were grown in minimal media to late mid-log phase, and vacuoles were imaged by fluorescence microscopy after labeling with FM4-64.

# Supplemental One



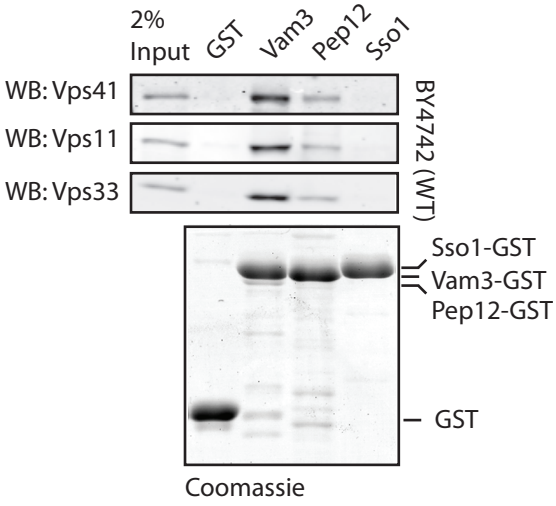
# Supplemental Two

Input: Purfied Vps33

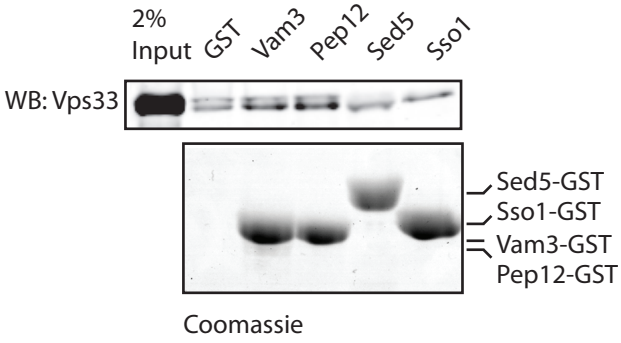


# Supplemental Three

A) Input: Cell Lysate

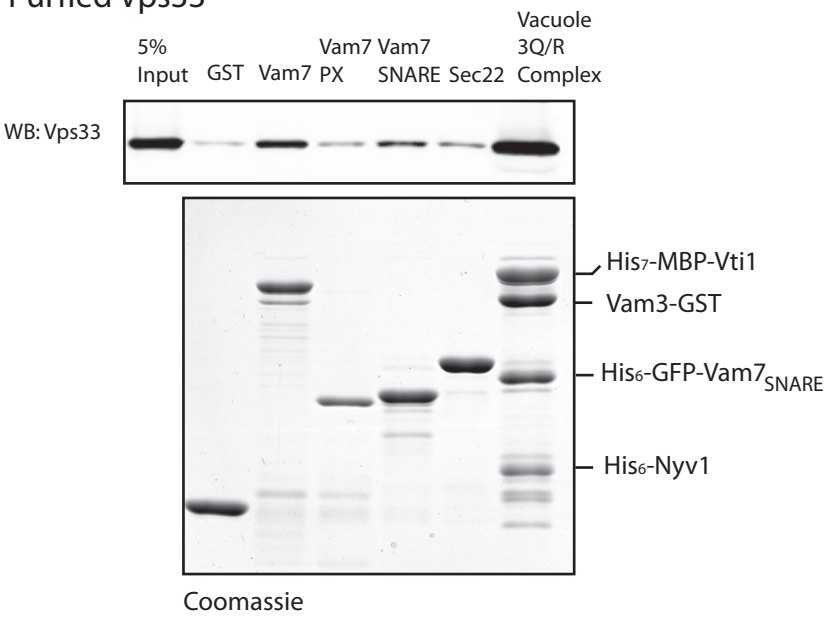



B) Input: Purified Vps33



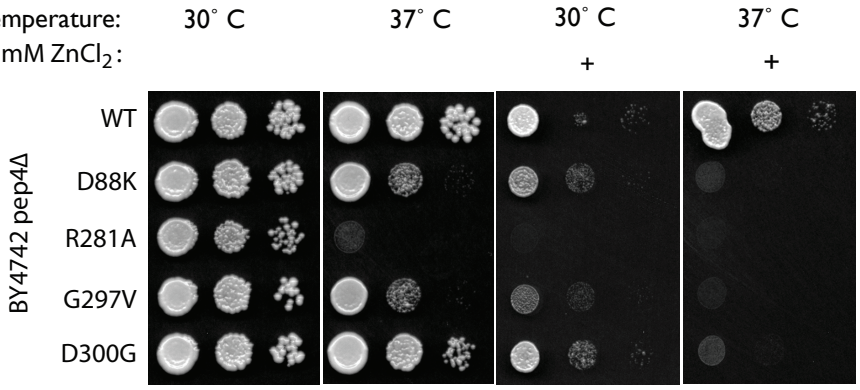
# Supplemental Four

Input: Purfied Vps33



# Supplemental Five

A)




B)