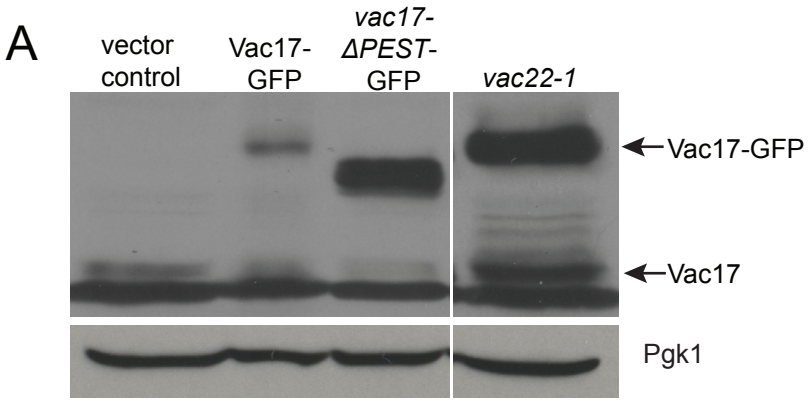
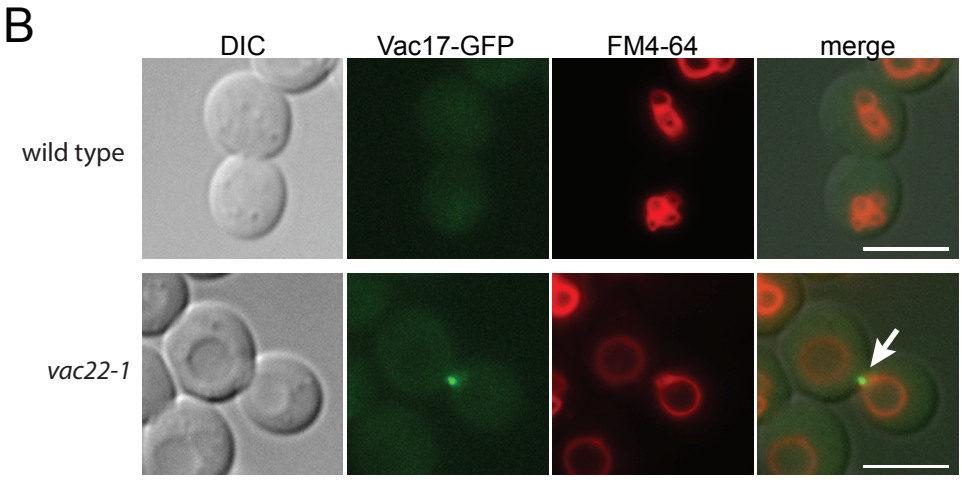
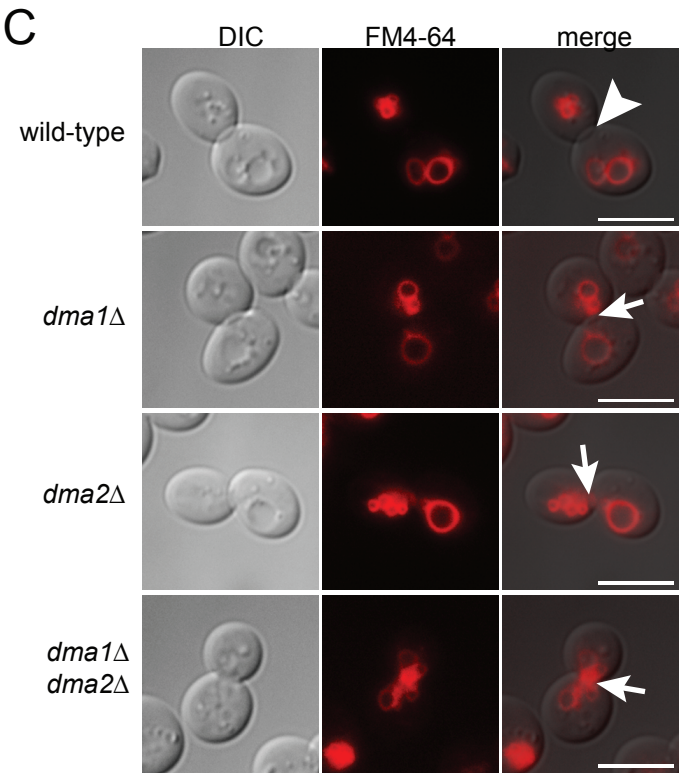
Figure S1


A
vector control
Vac17-GFP
vac17-ΔPEST-GFP
vac22-1
Vac17-GFP
Vac17
Pgk1


B
DIC
Vac17-GFP
FM4-64
merge
wild type
vac22-1


C
DIC
FM4-64
merge
wild-type
dma1Δ
dma2Δ
dma1Δ dma2Δ

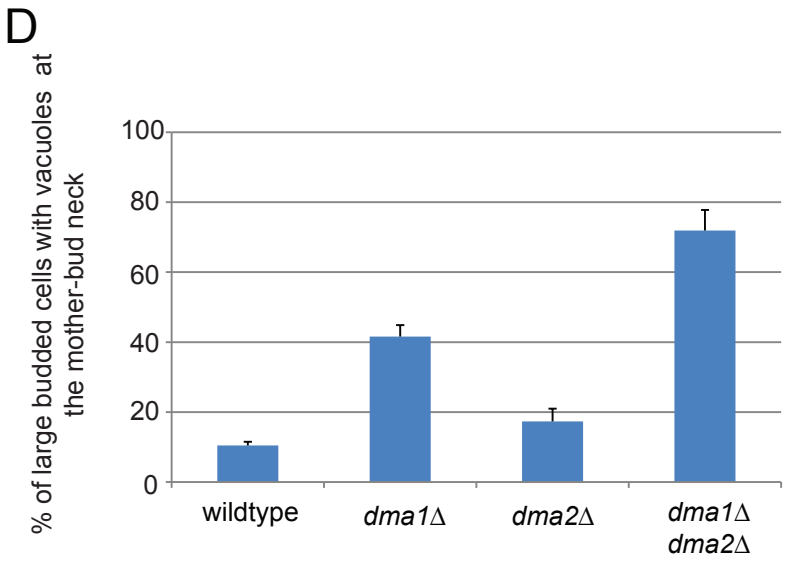

D
% of large budded cells with vacuoles at the mother-bud neck
100
80
60
40
20
0
wildtype
dma1Δ
dma2Δ
dma1Δ dma2Δ

Figure S2

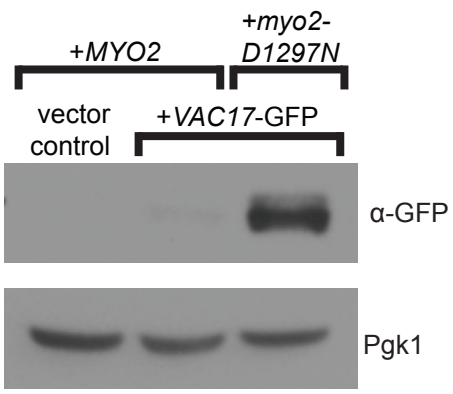


Figure S3

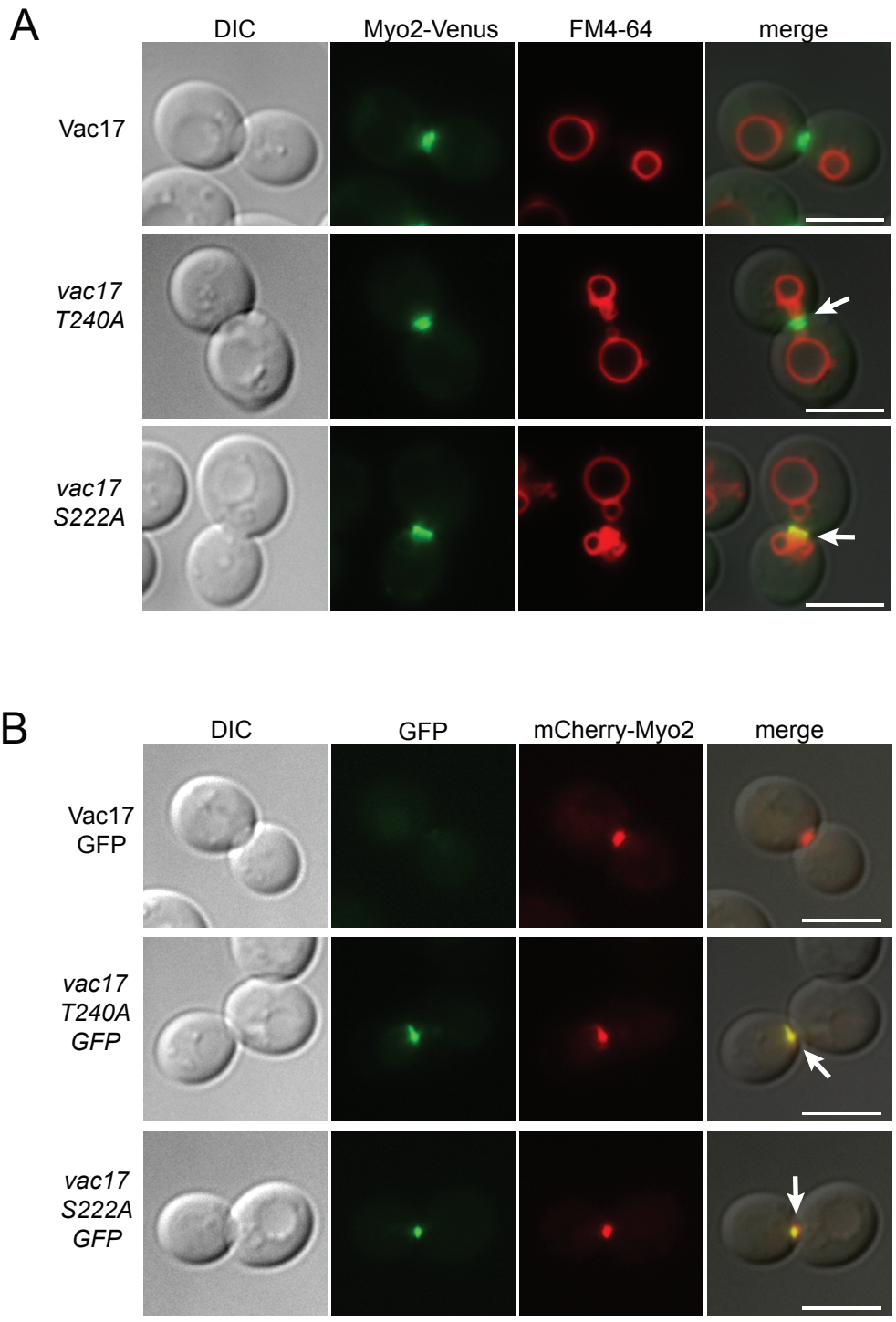
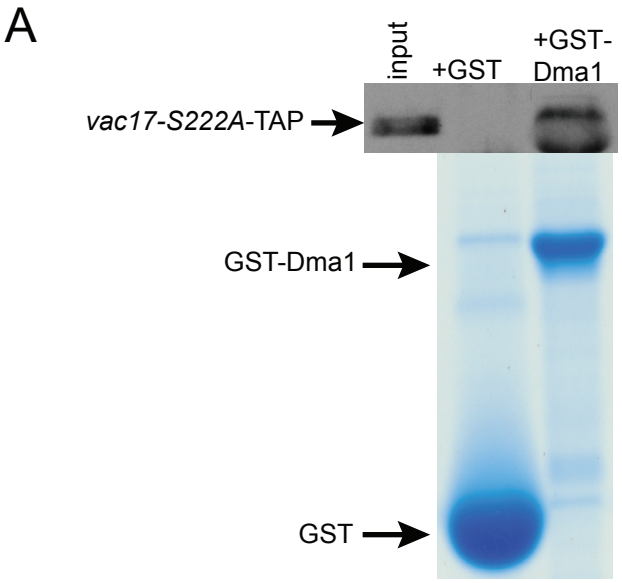
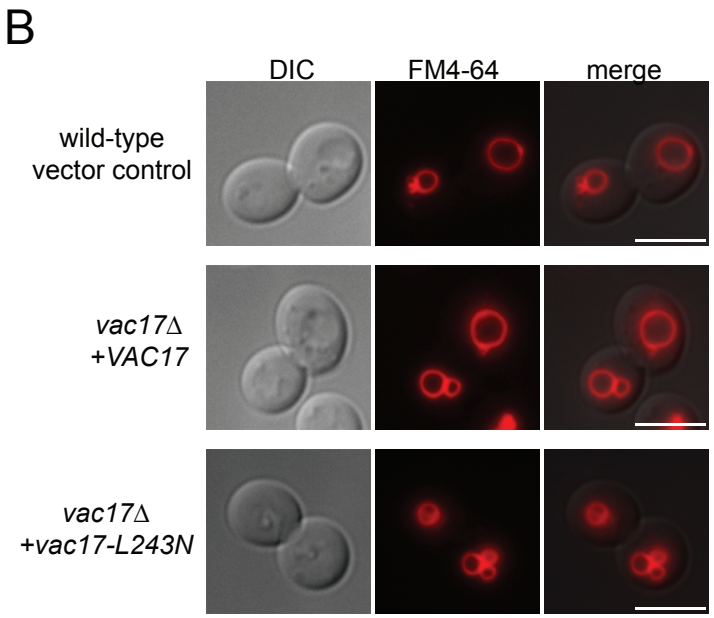

A
DIC
Myo2-Venus
FM4-64
merge
Vac17
vac17 T240A
vac17 S222A
B
DIC
GFP
mCherry-Myo2
merge
Vac17 GFP
vac17 T240A GFP
vac17 S222A GFP

Figure S4


A
input
+GST
+GST-Dma1
vac17-S222A-TAP
GST-Dma1
GST


B
DIC
FM4-64
merge
wild-type vector control
vac17Δ +VAC17
vac17Δ +vac17-L243N

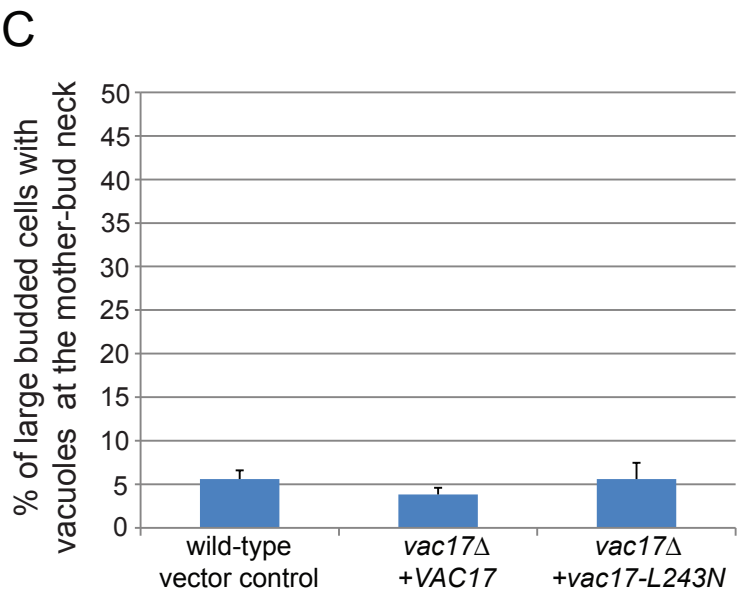

C
% of large budded cells with vacuoles at the mother-bud neck
50
45
40
35
30
25
20
15
10
5
0
wild-type vector control
vac17Δ +VAC17
vac17Δ +vac17-L243N

Figure S5

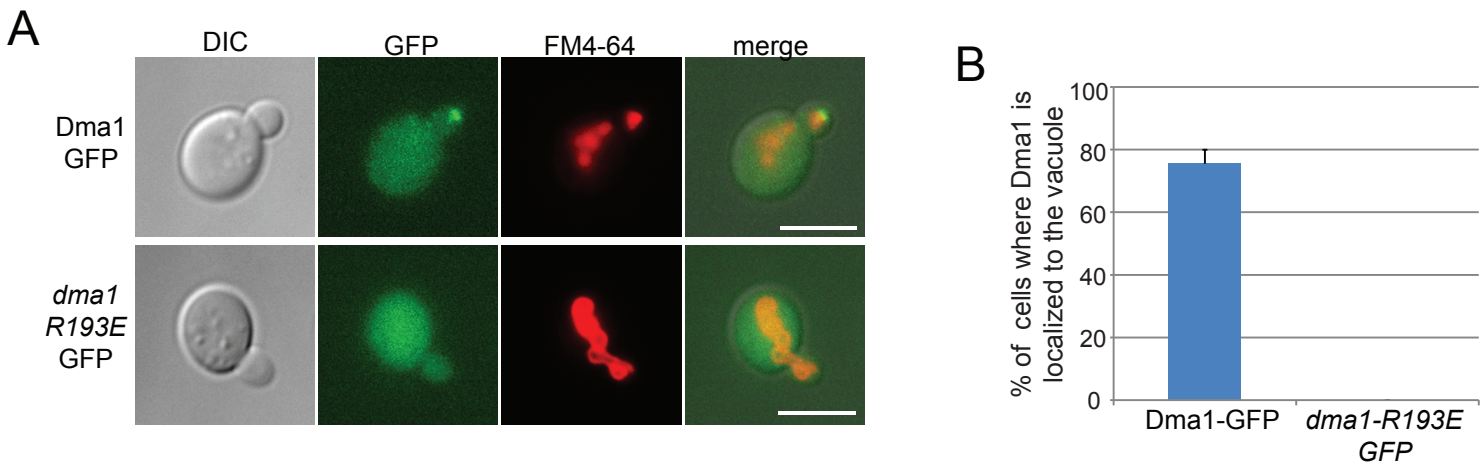
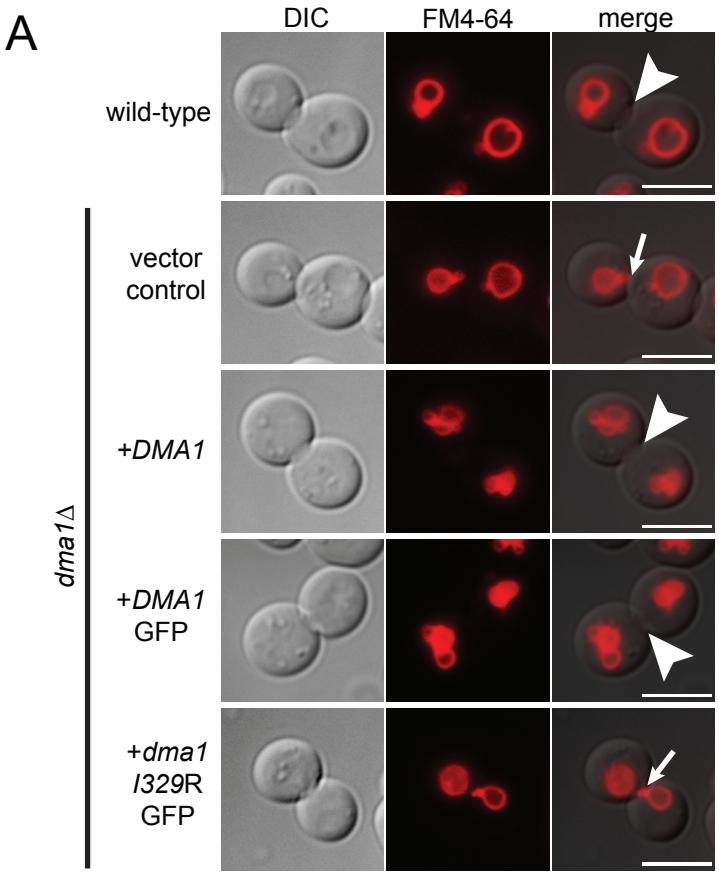
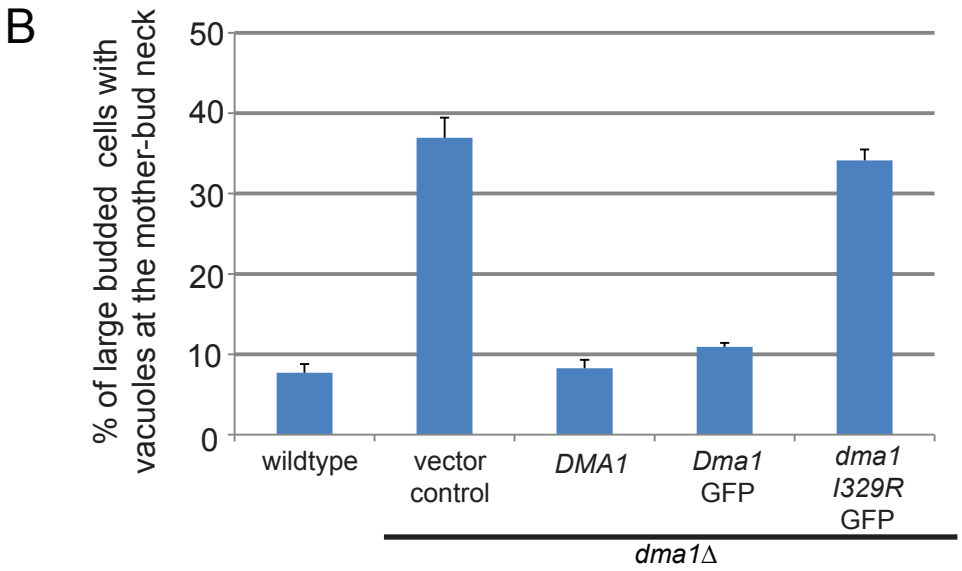
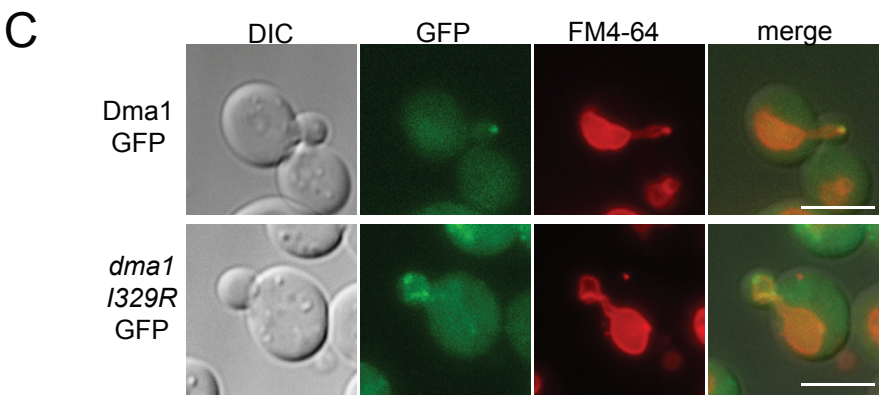

A
DIC
GFP
FM4-64
merge
Dma1
GFP
dma1
R193E
GFP
B
% of cells where Dma1 is localized to the vacuole
100
80
60
40
20
0
Dma1-GFP
dma1-R193E
GFP

Figure S6


A
DIC
FM4-64
merge
wild-type
vector control
+DMA1
+DMA1 GFP
+dma1 I329R GFP
dma1Δ


B
% of large budded cells with vacuoles at the mother-bud neck
50
40
30
20
10
0
wildtype
vector control
DMA1
Dma1 GFP
dma1 I329R GFP
dma1Δ


C
DIC
GFP
FM4-64
merge
Dma1 GFP
dma1 I329R GFP

**Supplemental Figure Legends**

**Figure S1, related to Figure 1. *DMA1* and *DMA2* are required for the detachment of the vacuole from Myo2.** (A) The *vac22-1* mutant has elevated levels of both Vac17-GFP and endogenous Vac17. (B) Vac17-GFP and the vacuole are inappropriately transported to the mother-bud neck in the *vac22-1* mutant (arrow). (C) In the *dma1Δ*, *dma2Δ* and double *dma1Δ dma2Δ* mutants, termination of vacuole transport is defective (arrows). (D) Percentage of large budded cells with vacuoles accumulated at the mother-bud neck in the mutants shown in (C). anti-Vac17 antibodies; 1:1,000 dilution. anti-Pgk1 antibodies; 1:5,000 dilution. Error bars; SEM. Bar = 5 μm.

**Figure S2, related to Figure 2. Vac17 levels are elevated when vacuole transport is blocked.** A *myo2Δ vac17Δ* mutant expressing either *MYO2* or *myo2-D1297N* with *VAC17*-GFP from plasmids. Expression of *myo2-D1297N*, a mutant which is defective in binding Vac17 and vacuole transport, Vac17-GFP levels are elevated compared to cells expressing *MYO2*. anti-GFP antibodies; 1:1,000 dilution. anti-Pgk1 antibodies; 1:5,000 dilution.

**Figure S3, related to Figure 3. *vac17* point mutants fail to dissociate from Myo2.** (A) In cells expressing *vac17-T240A* and *vac17-S222A*, but not *VAC17*, vacuoles colocalize with Myo2-Venus at the mother-bud neck in large budded cells (arrows). (B) *vac17-T240A*-GFP and *vac17-S222A*-GFP, but not Vac17-GFP, colocalize with mCherry-Myo2 at the mother-bud neck (arrows) in large budded cells. Bar = 5 μm.

**Figure S4, related to Figure 4. Characterization of Vac17 residues required for Dma1 to bind Vac17 and for the termination of vacuole transport.** (A) GST-Dma1, but not the GST tag alone, binds *vac17-S222A*-TAP in yeast lysates. (B) The termination of vacuole transport occurs normally in the *vac17-L243N* mutant. (C) Quantification of the percentage of large budded cells with vacuoles at the mother-bud neck in wild-type cells and the *vac17Δ* mutant expressing either *VAC17* or *vac17-L243N*. A minimum of 100 cells were counted per experiment for 3 experiments. Error bars; SEM. Bar = 5 μm.

**Figure S5, related to Figure 5. The FHA domain of Dma1 is required for the recruitment of Dma1 to the vacuole.** (A) The *dma1-R193E*-GFP mutant fails to localize to the bud vacuole. (F) Quantification of the percentage of small budded cells where Dma1-GFP or *dma1-R193E*-GFP localizes to the vacuole. A minimum of 20 cells were counted per experiment for 3 experiments. Z-sections of small budded cells were analyzed. Error bars; SEM. Bar = 5 μm.

**Figure S6, related to Figure 6. The E3 ubiquitin ligase activity of Dma1 is required for the termination of vacuole movement.** (A) Expression of *DMA1* and *DMA1*-GFP but not an enzymatically inactive mutant, *dma1-I329R*-GFP, or vector control rescues the termination of vacuole movement in the *dma1Δ* mutant. Arrowheads; vacuoles are deposited properly in the bud and do not accumulate at the mother-bud neck. Arrows; vacuoles are mis-targeted to the mother-bud neck. (B) Percentage of large budded cells with vacuoles accumulated at the mother-bud neck in the *dma1Δ* mutant expressing vector control, *DMA1*, *DMA1*-GFP or *dma1-I329R*-GFP. A minimum of 100 cells were

counted per experiment for 3 experiments. Error bars; SEM. (C) *dma1-I329R*-GFP localizes to a broader area on the bud vacuole. Bar = 5 μm.

**Table S1, related to Experimental Procedures.** Strains used in this study.

**Table S2, related to Experimental Procedures**. Plasmids used in this study.

Table S1, related to Experimental Procedures. Yeast Strains Used in this Study.

| Strain | Genotype | Source |
|---|---|---|
| LWY7235 | *MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9* | Catlett and Weisman, 1998 |
| LWY5798 | *MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, vac17Δ::TRP1* | Tang et al., 2003 |
| LWY7664 | *MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, vac17Δ::TRP1, MYO2-GFP::HIS3* | Peng and Weisman, 2008 |
| LWY12269 | *MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, GFP-TUB1::URA3, kar9Δ ::kan$^r$, SPC42-mCherry::HIS3* | This study |
| LWY12086 | *MATα, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, vac17Δ::TRP1, myo2Δ::TRP1 [pRS413-mCherry-MYO2]* | This study |
| LWY11102 | *MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, dma1Δ::kan$^r$* | This study |
| LWY11125 | *MATα, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, dma2Δ::kan$^r$* | This study |
| LWY11156 | *MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, dma1Δ::kan$^r$, dma2Δ::kan$^r$* | This study |
| LWY11269 | *MATα, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, vac17Δ::TRP1, dma1Δ::kan$^r$* | This study |
| LWY11389 | *MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, dma1Δ::kan$^r$, dma2Δ::kan$^r$, VAC17-TAP::LEU2* | This study |
| LWY11524 | *MATα, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, dma1Δ::kan$^r$, dma2Δ::kan$^r$, vac17-F225S-TAP::LEU2* | This study |
| LWY11528 | *MATα, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, dma1Δ::kan$^r$, dma2Δ::kan$^r$, vac17-L221P-TAP::LEU2* | This study |
| LWY11507 | *MATα, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, dma1Δ::kan$^r$, dma2Δ::kan$^r$, vac17-T240A-TAP::LEU2* | This study |
| LWY11541 | *MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, dma1Δ::kan$^r$, dma2Δ::kan$^r$, vac17-S222A-TAP::LEU2* | This study |
| LWY11687 | *MATα, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, dma1Δ::kan$^r$, dma2Δ::kan$^r$, vac17Δ::TRP1* | This study |
| LWY8195 | *MATa, ura3-52, leu2-3,-112, his3-Δ200, trp1-Δ901, lys2-801, suc2-Δ9, pep4-Δ1137, vac17Δ::TRP1, myo2Δ::TRP1 [YCp50-MYO2]* | This study |
| MHY501 | *MAT*α, *his3-Δ200, leu2-3, -112, ura3-52, lys2-801, trp1-1* | Chen and Hochstrasser, 1993 |

| | | |
|---|---|---|
| MHY1409 | *MATα, his3-Δ200, leu2-3, -112, ura3-52, lys2-801, trp1-1, uba1-2* | Swanson and Hochstrasser, 2000 |
| MHY605 | *MATα, his3-11, leu2-3, -112, ura3-Δ5, pre1-1::can$^{R}$* | Chen and Hochstrasser, 1996 |
| MHY952 | *MATα, his3-Δ200, leu2-3, -112, ura3-52, lys2-801, trp1-1, doa3-delta1::HIS3 [YEpDOA3$_{LS}$][YCpUbDOA3ΔLS]* | Arendt and Hochstrasser, 1997 |
| MHY973 | *MATα, his3-Δ200, leu2-3, -112, ura3-52, lys2-801, trp1-1, doa3-delta1::HIS3 [YEpDOA3$_{LS}$][YCpUbDOA3ΔLS-T76A]* | Arendt and Hochstrasser, 1997 |
| MHY1072 | *MATa, his3-Δ200, leu2-3, -112, ura3-52, lys2-801, trp1-1, pup1::leu2::HIS3, pup3-delta2::HIS3 [Ycplac22PUP1][Yeplac181PUP3]* | Arendt and Hochstrasser, 1997 |
| MHY1071 | *MATa, his3-Δ200, leu2-3, -112, ura3-52, lys2-801, trp1-1, pup1::leu2::HIS3, pup3-delta2::HIS3 [Ycplac22pup1-K58E][Yeplac181pup3-E151K]* | Arendt and Hochstrasser, 2007 |

Table S2, related to Experimental Procedures. Plasmids Used in this Study.

| Plasmid | Description | Source |
|---|---|---|
| pRS416-*DMA1* | CEN URA3 | This study |
| pRS416-*dma1-G232R* | CEN URA3 | This study |
| pRS416-*dma1-I329R* | CEN URA3 | This study |
| pRS416-*dma1-I329*-GFP | CEN URA3 | This study |
| pRS416-*DMA1*-GFP | CEN URA3 | This study |
| pRS416-*DMA1*-tdTomato | CEN URA3 | This study |
| pGEX4T-1 *DMA1* | Amp | This study |
| pGEX4T-1 *dma1-I329R* | Amp | This study |
| pRS413-mCherry-*MYO2* | CEN HIS3 | Jin et al., 2011 |
| pRS413-*MYO2*-Venus | CEN HIS3 | This study |
| pRS415-*VAC17* | CEN LEU2 | This study |
| pRS415-*VAC17*-GFP | CEN LEU2 | This study |
| pRS416-*vac17-S222A* | CEN URA3 | This study |
| pRS415-*vac17-S222A*-GFP | CEN LEU2 | This study |
| pRS415-*vac17-T240A* | CEN LEU2 | This study |
| pRS415-*vac17-T240A*-GFP | CEN LEU2 | This study |
| pGEX4T-1 Myo2 cargo-binding domain | Amp | Pashkova et al., 2006 |
| pRS416-*vac17-S202A* | CEN URA3 | This study |
| pRS416-*vac17-S206A,S207A,S208A* | CEN URA3 | This study |
| pRS416-*vac17-S213A* | CEN URA3 | This study |
| pRS416-*vac17-T224A* | CEN, URA3 | This study |
| pRS416-*vac17-S236A* | CEN URA3 | Stringer and Piper, 2012 |
| pRS416-*vac17-S247A,T248A* | CEN, URA3 | This study |
| pVT102-*VAC17*-GFP | 2μ URA3 | This study |
| pVT102-*VAC17* | 2μ URA3 | Eves et al., 2012 |

## Supplemental Experimental Procedures

### *In Vitro* Binding and Competition Experiments

Expression of GST tagged fusion proteins from BL21 star DE3 cells was induced with 0.4 mM IPTG (Denville Scientific); 16 $^{O}$C overnight. Cells were resuspended in 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 4 mM $MgCl_2$, 10% glycerol, 1 M NaCl, 5 mM DTT, 1 mM Pefabloc and Complete EDTA-free protease inhibitor cocktail (Roche) and lysed via sonication. An equal amount of lysis buffer without NaCl was added to the clarified lysates and incubated with glutathione Sepharose beads (GE Healthcare). Immobilized GST fusion proteins were washed with wash buffer (50 mM Tris-HCl pH 7.5, 4 mM $MgCl_2$, 10% glycerol, 0.5 M NaCl and 1 mM DTT and then with 50 mM HEPES-KOH pH 7.6, 150 mM KCl, 1 mM EDTA, 1 mM $Na_3VO_4$ and 10% glycerol). Yeast cells grown in YEPD at 24$^{O}$C were resuspended in 50 mM HEPES-KOH, pH 7.6, 150 mM KCl, 1 mM EDTA, 20 mM sodium pyrophosphate, 10 mM $NaN_3$, 20 mM NaF, 1 mM $Na_3VO_4$, 100 mM β-glycerophosphate, 0.5% OG, 10% glycerol, 1X Protease inhibitor cocktail (Sigma) and Complete EDTA-free protease inhibitor cocktail (Roche) and lysed with glass beads. GST and GST fusion protein bound beads were incubated with clarified yeast cell extracts for 1 hour at 4 $^{O}$C with agitation. Beads were then washed with wash buffer. Bound proteins were analyzed via SDS-page, Gelcode Blue staining (Thermo Scientific) and by immunoblot.

For competition experiments, GST-Dma1 bound beads were first incubated with 0.5 mg peptides resuspended in 50 mM HEPES-KOH pH 7.6, 150 mM KCl, 1 mM EDTA and 10% glycerol prior to incubation with yeast cell extracts.

### Immunoprecipitation Experiments

TCA precipitated proteins were pelleted and washed with acetone. The dried protein pellets were resuspended in 200 µl urea cracking buffer (6 M urea, 1% SDS, 50 mM Tris-HCl pH 7.5) and heated at 75 $^{O}$C for 10 min. 1.8 ml TWIP buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Tween-20, 0.1 mM EDTA) containing 1 mM $Na_3VO_4$ and 1X protease inhibitor cocktail (Sigma) was added to the resuspended protein. Undissolved proteins were pelleted via centrifugation. 4 µg of mouse anti-GFP antibodies (Roche) was added to the supernatant and incubated with agitation at 4 $^{O}$C, overnight. 50 µl of protein G beads (Sigma) washed with TWIP buffer was added and incubated with agitation at 4 $^{O}$C for 1 hour. Beads were collected via centrifugation and washed with TWIP buffer. Bound proteins were analyzed via immunoblot.

For dephosphorylation of Vac17-GFP, Protein G beads were collected via centrifugation and washed 3 times with TWIP buffer without EDTA. The beads were resuspended in 1X λ-ppase buffer containing 1X protease inhibitor cocktail (Sigma) and 10 mM $MnCl_2$. Either water, λ-ppase (400 units, New England Biolabs) or λ-ppase plus phosphatase inhibitors (100 mM NaF, 10 mM $Na_3VO_4$, 50 mM EDTA, 20 mM β-glycerophosphate and 20 mM sodium pyrophosphate) were added to the samples. Phosphatase reactions were performed in a volume of 100 µl and incubated at 30 $^{O}$C for 1 hour. Reactions were terminated by addition of 50 µl 2X SDS sample buffer and heated at 75 $^{O}$C for 10 min.

**Thermostability Measurements**

Expression of GST tagged fusion proteins from BL21 star DE3 cells was induced with 0.1 mM IPTG (Denville Scientific); 16 $^{O}$C overnight. Cells were harvested and

resuspended in 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 1 mM Pefabloc and Complete EDTA-free protease inhibitor cocktail (Roche) and lysed via sonication. Triton X-100 was added to a final concentration of 1%. Clarified lysates were incubated with glutathione Sepharose beads (GE Healthcare) and washed with lysis buffer containing 0.01% Triton X-100. Bound proteins were eluted in wash buffer containing 25 mM glutathione and 50 mM NaOH. Recombinant proteins were then purified on a size exclusion column (HiLoad 16/60 Superdex 200; GE Healthcare). Protein unfolding was measured using a ThermoFluor plate reader (Johnson and Johnson). Samples containing ~0.2 mg/ml protein, 1 mM peptide and 100 µM 8-Anilino-1-naphthalenesulfonic acid (ANS, Sigma) were prepared in a 384-well plate and fluorescence was measured from 15 $^{O}$C to 80 $^{O}$C in 1 $^{O}$C increments. Melting temperatures were determined automatically using the Thermofluor Acquire software.

## Supplemental References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