Spatial regulation of organelle release from myosin V transport by p21-activated kinases

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Correct positioning of organelles is essential to eukaryotic cells. Molecular motors transport organelles to their proper destinations, yet little is known about the pathways that define these destinations. In Saccharomyces cerevisiae, the myosin V motor Myo2 binds the vacuole-specific adapter Vac17 to attach to the vacuole/lysosome and initiate transport. After arrival in the bud, Myo2 releases the vacuole, and Vac17 is degraded. However, the mechanisms that spatially regulate this release were not established. In this study, we report that the bud cortex is a landmark that signals a successful delivery of the vacuole to the bud. We demonstrate that upon arrival at the bud cortex, Vac17 is phosphorylated by Cla4. Cla4-dependent phosphorylation is required for the ubiquitylation and subsequent degradation of Vac17 and the release of the vacuole from Myo2. Our study reveals a critical step in the spatial regulation of myosin V–dependent organelle transport and may reveal common mechanisms for how molecular motors accurately deposit cargoes at the correct locations.

Introduction

Myosin V motors transport organelles to their correct intracellular locations. Transport initiates when myosin V binds cargo-specific adapters and attaches to organelles (Weisman, 2006; Westermann, 2014; Knoblach and Rachubinski, 2016). Upon arrival at their correct locations, organelles detach from myosin V, thereby terminating transport. The molecular mechanisms that signal the arrival of organelles at their correct locations remain unclear.

At the beginning of the cell cycle in Saccharomyces cerevisiae, the myosin V motor Myo2 binds the vacuole adapter Vac17 and transports a portion of the mother vacuole into the bud (Ishikawa et al., 2003; Peng and Weisman, 2008; Eves et al., 2012). In the bud, the vacuole is released from Myo2, and Vac17 is degraded (Tang et al., 2003). Degradation of Vac17 requires phosphorylated Vac17-T240, which recruits the E3 ubiquitin ligase Dma1. Dma1 then ubiquitylates Vac17, targeting Vac17 for degradation by the proteasome (Yau et al., 2014). Late in the cell cycle, Myo2 transports secretory vesicles, but not the vacuole, to the mother-bud neck (Govindan et al., 1995; Pryne et al., 1998; Schott et al., 1999). In mutants defective in the degradation of Vac17, the vacuole remains attached to Myo2 and is inappropriately transported to the mother-bud neck late in the cell cycle (Tang et al., 2003; Yau et al., 2014). In yeast, the p21-activated kinases (PAKs) Cla4 and Ste20 localize to the bud cortex and regulate several cell cycle–related processes, including actin cytoskeleton polarization, septin ring assembly, and cytokinesis (Cvrcková et al., 1995; Peter et al., 1996; Kumar et al., 2009; Boyce and Andrianopoulos, 2011). Moreover, it was proposed that PAKs indirectly regulate vacuole inheritance via Lte1, a Cla4-activated guanine nucleotide exchange factor (Bartholomew and Hardy, 2009).

In this study, we report that PAKs directly phosphorylate Vac17 to signal the arrival of the vacuole at its correct location. We show that before the termination of vacuole inheritance, the vacuole extends to the bud cortex, where Vac17 colocalizes with Cla4. In contrast to a previous study, we find that neither degradation of Vac17 nor termination of vacuole transport require Lte1 (Bartholomew and Hardy, 2009). Instead, Cla4 directly phosphorylates Vac17-S222. This phosphorylation event is required for Dma1-dependent ubiquitylation and degradation of Vac17 as well as the release of the vacuole from Myo2. Moreover, in a Vac17 mutant that cannot be phosphorylated at S222, the vacuole remains attached to the bud cortex, which suggests that this is the intracellular location where Cla4 phosphorylates Vac17. Collectively, these studies suggest that at the bud cortex, Cla4 initiates a signaling cascade to regulate the ubiquitylation of Vac17 and complete the termination of vacuole transport, thereby ensuring that the vacuole is deposited at the correct intracellular location.

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Abbreviation used: PAK, p21-activated kinase.
**Results and discussion**

**PAKs are required for Vac17 degradation and termination of vacuole transport**

A previous study suggested that Lte1 regulates Vac17 degradation (Bartholomew and Hardy, 2009). However, it was unknown whether Lte1 directly regulates the termination of vacuole transport. To investigate whether Lte1 acts in this pathway, we tested the *lte1Δ* mutant for the stabilization of Vac17-GFP and the mislocalization of Vac17-GFP and the vacuole to the mother-bud neck in large-budded cells. However, these defects were not observed in the absence of *LTE1* (Fig. 1, A–C). We then measured the impact of PAK function on vacuole inheritance. Vac17 levels were not detectably elevated in the *cla4Δ* or *ste20Δ* mutants. Furthermore, only a partial defect in the termination of vacuole was observed, whereby Vac17-GFP and the vacuole colocalized at the bud tip and mother-bud neck in some large-budded cells. These phenotypes were more prominent in the *cla4Δ* mutant (Fig. 1 D–F).

Because PAKs are functionally redundant and synthetically lethal, we performed experiments in the *cla4Δ ste20Δ* double mutant (Cvrcková et al., 1995; Martin et al., 1997; Holly and Blumer, 1999; Tatebayashi et al., 2006). At 24°C, the levels of Vac17 in the *ste20Δ cla4Δ* mutant were similar to wild-type levels. In contrast, at 37°C, Vac17 was stabilized and exhibited an increase in electrophoretic mobility, which suggests a loss of posttranslational modifications (Fig. 1 A). In the *cla4Δ ste20Δ* mutant at 24°C, there was partial mislocalization of the vacuole with Vac17-GFP, consistent with the finding that the *ste20Δ* mutant has a minor defect in the termination of vacuole transport (Fig. 1, S1, D–F). In large-budded *cla4Δ ste20Δ* cells at 37°C, Vac17-GFP and the vacuole accumulated at the mother-bud neck, similar to the *dma1Δ* *dma2Δ* mutant (Yau et al., 2014). Intriguingly, we also observed Vac17 and the vacuole at a new aberrant location: the bud tip. This raises the possibility that the bud cortex is the landmark where Myo2 releases the vacuole. In addition, Vac17-GFP and the vacuole mislocalized to the cell cortex at a site adjacent to the mother-bud neck (a location on the cortex between the bud tip and mother-bud neck). This localization had not been previously reported for the vacuole or Myo2 (Fig. 1, B and C). The mislocalization of the vacuole to this site may be caused by defects in the organization of the actin cytoskeleton in the *cla4Δ ste20Δ* mutant (Holly and Blumer, 1999).

To test whether PAK function is required to detach the vacuole from Myo2, we analyzed colocalization between Myo2-Venus and the vacuole in large-budded cells. In wild-type cells at 24°C and 37°C, the vacuole detached correctly and did not colocalize with Myo2-Venus. In the *cla4Δ ste20Δ* mutant at 24°C, there was a modest defect in the termination of vacuole transport. At 37°C in the *cla4Δ ste20Δ* mutant, there was a strong defect in the detachment of the vacuole from Myo2-Venus. The vacuole colocalized with Myo2-Venus at the bud tip, mother-bud neck, and adjacent to the mother-bud neck (Fig. 1, D and E). These observations suggest that PAK-dependent signaling regulates Vac17 degradation, the release of the vacuole from Myo2, and the termination of vacuole transport.

**Cla4 phosphorylates Vac17 in vivo and in vitro**

That PAKs regulate Vac17 levels independently of Lte1 suggests that PAKs directly target Vac17. In support of this hypothesis, recombinant GST-Cla4, but not GST alone, binds Vac17-TAP from cell extracts (Fig. 2 A). Cla4 phosphorylates serines within the consensus motif RXS (Wu et al., 1996; Versele and Thorner, 2004; Mok et al., 2010). Interestingly, Vac17-S222 matches this motif, Vac17-R220LS222, and is required for Vac17 degradation and the termination of vacuole transport (Yau et al., 2014). To determine whether Vac17-S222 is a Cla4 phosphorylation site, we generated a phosphospecific antibody for Vac17-pS222 and tested it against Vac17-GFP and vac17-S222A-GFP expressed in *vac17Δ* or *dma1Δ dma2Δ vac17Δ* mutants. Deletion of DMA1 and DMA2 stabilizes phosphorylated Vac17, thereby facilitating its detection (Yau et al., 2014). The anti-pS222 antibody recognized Vac17-GFP but not vac17-S222A-GFP (Fig. 2 B). Furthermore, this antibody does not recognize dephosphorylated Vac17-GFP, indicated by an increase in electrophoretic mobility, in λ-phosphatase–treated samples (Fig. 2 C). These results demonstrate the specificity of this antibody for Vac17-pS222 and that Vac17-S222 is phosphorylated in vivo.

To test whether PAK activity is required for the phosphorylation of Vac17-S222 in vivo, we analyzed Vac17-S222 phosphorylation in the *cla4Δ ste20Δ* mutant. Inactivation of PAK activity reduced phosphorylation of Vac17-S222. This result suggests that PAK activity is required for the phosphorylation of S222 in vivo. Phosphorylation of T240 may have been slightly affected by reduced pS222 or by indirect effects of the *cla4Δ ste20Δ* mutant (Fig. 2, D and E).

Because Vac17-S222 matches the Cla4 consensus site, we tested whether Cla4 directly phosphorylates Vac17. We performed in vitro kinase assays using recombinant GST-Cla4 and a 6xHIS-Vac17 (96–355) peptide. Phosphorylation was detected via immunoblotting with the anti-pS222 antibody. GST-Cla4, but not kinase-dead GST-cla4-K594A, phosphorylated 6xHIS-Vac17 (96–355) in an ATP-dependent manner. Additionally, the antibody did not recognize products of this reaction if the 6xHIS-Vac17-S222A (96–355) peptide was used (Fig. 2 F). Collectively, these results demonstrate that Cla4 directly phosphorylates Vac17-S222.

**Phosphorylation of Vac17-S222 is required for the termination of vacuole transport**

To gain insight into the role of Vac17-pS222, we tested the termination of vacuole transport in cells expressing the nonphosphorylatable vac17-S222A-GFP mutant. In large-budded cells, vac17-S222A-GFP mislocalized with the vacuole at the mother-bud neck, similar to vac17-T240A-GFP. Upon closer analysis, both vac17Δ mutants also accumulated with the vacuole at the bud tip in large-budded cells before relocating to the mother-bud neck, as seen in the *cla4Δ ste20Δ* mutant (Fig. 3, A and B). These results demonstrate that phosphorylation of Vac17-S222 is required to terminate vacuole transport and support the hypothesis that termination of transport initiates at the bud tip. In addition, the localization of Myo2 was not perturbed by the mislocalization of the vacuole (Fig. 3, C–E) or Vac17 (Fig. 3, F–H) in the vac17-S222A-GFP and vac17-T240A-GFP mutants. This suggests that the trajectory of Myo2 is not dictated by its bound cargoes.

**Vac17-pS222 is required for the ubiquitylation of Vac17**

The termination of vacuole transport occurs in regulated steps: (A) Vac17-T240 is phosphorylated, (B) Dma1 is recruited to the vacuole, (C) Dma1 ubiquitylates Vac17, and (D) Vac17 is
PAKs are required for the degradation of Vac17 and the release of the vacuole from Myo2. (A) Vac17 levels are elevated in dma1Δ and cla4Δ ste20Δ mutants. The cla4Δ ste20Δ mutant was grown at either 24°C or shifted to 37°C for 3 h before lysis. Pgk1 was used as a loading control. Molecular mass is shown in kilodaltons. (B–E) Loss of PAK function results in mislocalization of the vacuole (FM4-64; B and D) and accumulation of Vac17-GFP (B) at the bud tip (arrowheads) or mother-bud neck (arrows). Wild-type (WT) and cla4Δ ste20Δ cells were transformed with Vac17-GFP (B) or Myo2-Venus (D). After FM4-64 labeling, cells were chased either at 24°C for 3 h or 24°C for 90 min and then 37°C for 90 min before imaging. DIC, differential interference contrast. (C and E) Quantification of >35 large-budded cells per condition per n. Error bars indicate SEM. n = 3. *, P < 0.05; **, P < 0.01; two-tailed Student’s t test.
degraded to release the vacuole from Myo2 (Yau et al., 2014). Because Vac17-S222 is required for the termination of vacuole transport, we tested whether Vac17-S222 functions at any of these known steps. Immunoblotting with the anti-pT240 antibody demonstrated that Vac17-GFP and vac17-S222A-GFP but not vac17-T240A-GFP were phosphorylated at T240. Conversely, Vac17-GFP and vac17-T240A-GFP but not vac17-S222A-GFP were phosphorylated at S222 (Fig. 4, A and B). These results demonstrate that phosphorylation of Vac17-S222 and Vac17-T240 occur independently of each other and are
Figure 3. **Termination of vacuole transport initiates at the bud cortex.** [A, C, and F] Fluorescence microscopy of a vac17Δ strain transformed with plasmids encoding GFP-tagged VAC17 or vac17 point mutants with or without mCherry- or Venus-tagged Myo2. [A] vac17-S222A-GFP and vac17-T240A-GFP resulted in mislocalization of the vacuole (FM4-64) to the bud tip (arrowheads) or mother-bud neck (arrows). [B] Quantification of >30 large-budded cells per strain per n. [C] Vacuoles colocalize with Myo2-Venus at the bud tip (arrowheads) or mother-bud neck (arrows) in cells expressing vac17-S222A or vac17-T240A. [D] Quantification of >40 large-budded cells for the colocalization of Myo2 and the vacuole. [E] Quantification of >40...
consistent with the trend of reduced Vac17-S222 phosphorylation during PAK inactivation (Fig. 2, D and E).

To determine whether Vac17-S222 is required for the recruitment of Dma1, we tested the localization of Dma1 during vacuole transport. We observed Dma1-3×GFP at the bud vacuole in the majority of small-budded cells expressing vac17-S222A, similar to wild type. This contrasts with the vac17-T240A mutant, which is defective in Dma1 recruitment (Fig. 4, C and D). That Dma1 is recruited to the vacuole in cells expressing vac17-S222A is consistent with the observation that vac17-S222A does not impair phosphorylation of Vac17-T240.

Because recruitment of Dma1 to the vacuole is unperturbed in the vac17-S222A mutant, we predicted that vac17-S222A would be ubiquitylated similarly to wild type. To test this hypothesis, VAC17-GFP or vac17-S222A-GFP were over-expressed in vac17Δ cells along with myo2-D1297N, a mutant defective in binding Vac17 (Ishikawa et al., 2003). The myo2-D1297N mutant was included because overexpression of vac17-S222A-GFP likely interfered with the ability of Myo2 to transport essential cargoes (Eves et al., 2012) and did not yield viable cells. Cells were also transformed with a plasmid encoding Myc-ubiquitin driven under an inducible promoter. GFP-tagged Vac17 constructs were immunoprecipitated, and ubiquitylation was detected via immunoblotting with anti-Myc antibodies. Surprisingly, vac17-S222A-GFP was not ubiquitylated in vivo (Fig. 4 E). These findings indicate that phosphorylation of Vac17-S222 is not required for the recruitment of Dma1 to the vacuole transport complex, but must occur before Dma1 can ubiquitylate Vac17. Thus, phosphorylation of Vac17-S222 may spatially regulate the termination of vacuole transport through regulating Dma1 activity.

### Cla4 signaling at the bud cortex initiates the release of the vacuole from Myo2

Cla4 localizes to the bud cortex of small-budded cells and later appears as a punctum on the vacuole in large-budded cells (Bartholomew and Hardy, 2009). To further analyze Cla4 localization in relation to vacuole transport, we performed time-lapse microscopy of cells expressing Cla4-3×GFP and the vacuole marker Vph1-mCherry. In small-budded cells, Cla4-3×GFP appears on the bud cortex, as previously described (Bartholomew and Hardy, 2009). A portion of the vacuole then enters the bud and extends to the bud cortex, where it colocalizes with Cla4. A punctum of Cla4 then moves onto the vacuole (Fig. S2). These observations suggest that arrival of the vacuole at the bud cortex initiates Cla4 signaling on the vacuole. Additionally, Cla4 activity is required for proper unloading of the vacuole from Myo2. In a wild-type cell, Vac17-GFP is barely visible, and the vacuole is in the center of the cell (Video 1). In the nonphosphorylatable vac17-S222A mutant, the vacuole and vac17-S222A-GFP persist at the bud tip and eventually move from the bud tip to the mother-bud neck (Video 2). In further support that Cla4 is acting at the cortex, we find that the Cla4 homologue Ste20 partially substitutes for Cla4 (Fig. S1, D and E), yet Ste20 is solely at the bud cortex (Peter et al., 1996; Takahashi and Pryciak, 2007). Collectively, these findings suggest that PKA signals the release of the vacuole from Myo2 at the bud cortex.

To determine whether Cla4 and Vac17 colocalize at the bud cortex, we analyzed the localization of Cla4-tdTomato and Vac17-GFP expressed in the cla4Δ vac17A mutant via synchronization with α-factor and imaging at 10-min intervals. At 50 min after release, Cla4-tdTomato and Vac17-GFP colocalized at the bud cortex. At 80 min after release, Vac17-GFP was no longer detected. This observation is consistent with the degradation of Vac17 (Tang et al., 2003; Peng and Weisman, 2008). Additionally, even though Vac17 was degraded, a punctum of Cla4 was observed on the vacuole as previously described (Figs. 5 A and S3; Bartholomew and Hardy, 2009). We then analyzed the dynamics of Cla4-tdTomato and vac17-S222A-GFP. In contrast to wild type, Cla4 and Vac17 colocalized 60 min after release. At 80 min after release, vac17-S222A-GFP accumulated with the vacuole at the bud tip, whereas a punctum of Cla4 localized to a different area of the vacuole. At 120 min after release, vac17-S222A-GFP mislocalized with the vacuole at the mother-bud neck (Figs. 5 B and S3). These data suggest that a transient interaction between Cla4 and Vac17 at the leading edge of the vacuole occurs after the vacuole reaches the bud cortex. Moreover, when Cla4 fails to phosphorylate vac17-S222A, vac17-S222A and the vacuole persist at the bud cortex before mislocalizing to the mother-bud neck (Fig. 5 B and Video 2). These data suggest that Cla4 phosphorylates Vac17-S222 upon arrival of the vacuole at the bud cortex, triggering Vac17 degradation and release of the vacuole from Myo2.

We report here that Cla4 provides spatial control for the termination of vacuole transport and that the bud cortex is the landmark that signals the successful delivery of a myosin V cargo to its correct intracellular location. We show that contact between Cla4 and Vac17 at the bud cortex (Fig. S2) initiates the termination of vacuole transport. Cla4 directly regulates Vac17 degradation via phosphorylating Vac17-S222 (Fig. 2). Intriguingly, phosphorylation by Cla4 is required for the ubiquitylation and degradation of Vac17 (Figs. 3 and 4) but not for the recruitment of Dma1 to the vacuole (Fig. 4). Collectively, our study suggests that once the vacuole reaches the bud cortex, Cla4 initiates a signaling cascade that activates Dma1 to ubiquitylate Vac17 and complete the termination of vacuole inheritance. Vac17-S222 phosphorylation may change the conformation of Vac17 so that it is amenable for Dma1-dependent ubiquitylation or recruit a binding partner that activates Dma1 (Fig. 5 C). These roles for Cla4 in Myo2 transport suggest a direct molecular link between cell polarity factors and the positioning of organelles. Our study reveals that myosin V relies on a spatially regulated signaling cascade to successfully transport cargoes to their correct destinations.

### Materials and methods

#### Yeast strains, plasmids, and media

Unless specified, yeast cultures were grown in yeast extract peptone dextrose containing 1% yeast extract, 2% peptone, and 2% dextrose or in synthetic complete media lacking the indicated amino acids at 24°C. Yeast strains and plasmids are listed in Tables S1 and S2, respectively.

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large-budded cells per strain per n for the localization of Myo2. [f] vac17-S222A-GFP and vac17-T240A-GFP colocalize with mCherry-Myo2 at the bud tip (arrowheads) or mother-bud neck (arrows). DIC, differential interference contrast. [G] Quantification of >58 large-budded cells per strain per n for the colocalization of Myo2 and Vac17. [H] Quantification of >58 large-budded cells per strain per experiment for the localization of Myo2. Error bars indicate SEM. n = 3. *, P < 0.05; **, P < 0.01; two-tailed Student’s t test.
**Western blot analysis**

Cells were harvested and lysed in 1 ml 0.2 M NaOH and 0.2% β-mercaptoethanol. Proteins were precipitated via the addition of 100 µl TCA and centrifuged at 12,000 rpm. Proteins were resuspended in 120 µl 2× SDS sample buffer followed by the addition of 30 µl of 1 M Tris base, pH ∼11. Samples were heated at 75°C and analyzed via immunoblot (Peng and Weisman, 2008). For immunoblot analyses, mouse anti-GFP (1:1,000; Roche), rabbit anti-TAP (1:1,000; Thermo Fisher Scientific), mouse anti-Pgk1 (1:10,000; Invitrogen), sheep anti-Vac17 (1:1,000; custom made; 21st Century Biochemicals), rabbit anti–phospho-Thr240 (1:2,500; custom made; 21st Century Biochemicals), and rabbit anti–phospho-Ser222 (1:2,500; custom
Immunoprecipitation experiments

Cells were lysed and proteins were precipitated as described in the Western blot analysis section. Precipitated proteins were washed with acetone, dried, and resuspended in 200 µl urea buffer (6 M urea, 1% SDS, and 50 mM Tris-HCl, pH 7.5) and then were heated at 75°C for 10 min. 1.8 ml TWIP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Tween-20, and 0.1 mM EDTA) containing 1 mM Na3VO4, and 1× protease inhibitor cocktail (Sigma-Aldrich) was added to the resuspended protein and centrifuged. 4 µg of mouse anti-GFP antibodies (Roche) were added to the supernatant and incubated with agitation at 4°C overnight. Immune complexes were harvested via the addition of protein G beads, which were subsequently 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 three times with TWIP buffer without EDTA. The beads were resuspended in 1× λ-phosphatase buffer containing 1× protease inhibitor cocktail (Sigma-Aldrich) and 10 mM MnCl2. Either water, λ-phosphatase (400 U; New England Biolabs, Inc.) or λ-phosphatase plus phosphatase inhibitors (100 mM NaF, 10 mM Na3VO4, 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°C for 1 h. Reactions were terminated by addition of 50 µl 2× SDS sample buffer and heated at 75°C for 10 min.
Antibody preparation
The anti-pT240 and anti-pS222 phosphospecific antibodies were made by 21st Century Biochemicals. To generate the anti-pT240 antibody, the unmodified peptide Thr240 NP (CKK-Ahx-FDSQDTIILPSLTFDE-amide) was used for both immunization and affinity purification. To generate the anti-pS222 antibody, the unmodified peptide Ser222 NP (C-Ahx-NKNQRLSLTFDE-amide) was used for immunodepletion. The immunogens pSer222 NT (C-Ahx-NKNQRL[pS]LTFDE-amide) and pSer222 CT (acytel-KYNRQL[pS]LTFDE-Ahx-C-amide) were mixed for both immunization and affinity purification.

To purify Vac17 antibodies, serum was loaded onto a protein A sepharose column equilibrated in 100 mM Tris-HCl, pH 8, and washed with 100 mM Tris-HCl, pH 8, followed by 10 mM Tris-HCl, pH 8. Antibodies were eluted with 100 mM glycine, pH 3, and equilibrated with 1 M Tris-HCl, pH 8. Elutions were then run through a total protein column made from vac17Δ lysates. To prepare the total protein column, 800 ml vac17Δ culture was heated at 50°C in Thunber buffer (5% SDS, 8 M urea, 100 mM Tris, pH 7, 2 mM EDTA, 5 mM β-mercaptoethanol, and 10% glycerol) and lysed with glass beads. Proteins were precipitated with acetone, resuspended in PBS/1% SDS, and applied to an Actigel ALD Ultraflow 4 column (Sterogene Bioseparations, Inc.) and then prepared according to the manufacturer’s protocols. The column was washed with 0.1 M NaHCO3, pH 8.5/0.5 M NaCl followed by 0.2 M glycine, pH 2.5, equilibrated with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20 (TTBS) containing 0.5 M NaCl, and then washed with TTBS/0.5 M NaCl/1× protease inhibitor cocktail (Sigma-Aldrich)/40 µM chymostatin. The IgG fraction was incubated in the column overnight, and flowthrough was then applied to a Vac17 affinity column. To construct the Vac17 affinity column, recombinant His-vac17Δ (97–260) was purified and applied to a Ni-Nta agarose column in PP lysate buffer, pH 7.5 (20 mM NaHPO4, 0.5 M NaCl, 10 mM imidazole, and 0.05% β-mercaptoethanol). IgG was loaded onto the column and washed with TTBS/0.5M NaCl. Fractions were eluted in 0.2 M glycine, pH 2.5, and equilibrated in 0.1 M Tris base.

In vivo ubiquitylation experiments
To detect ubiquitylated Vac17, pVT102U vectors encoding Vac17-GFP and vac17-S222A-GFP were transformed into a vac17Δ or dma1Δ vac17Δ mutant strain. A plasmid encoding Myc-ubiquitin expression was driven by a CUP1 promoter and the pRS413-Δvac17Δ mutant strain. A plasmid encoding Myc-ubiquitin Δvac17Δ was cotransformed into the same strain. Myc-ubiquitin expression was terminated via the addition of sample buffer and then heated at 75°C for 10 min. Proteins were resolved via SDS-PAGE and analyzed by Gelcode blue staining (Thermo Fisher Scientific) and immunoblot.

In vitro binding experiments
GST and GST fusion proteins were expressed in BL21 star DE3 cells via induction with 0.1 mM IPTG at 16°C overnight. Cells were lysed, and GST fusion proteins were immobilized on glutathione beads as described in the In vitro kinase experiments section. Immunoblotted proteins were washed once with yeast lysis buffer containing 0.5 µg kinase, 2 µg substrate, 10 mM ATP, and 50 mM β-glycerophosphate in a volume of 50 µl and then incubated at 30°C for 1 h. Kinase reactions were terminated via the addition of sample buffer and then heated at 75°C for 10 min. Proteins were resolved via SDS-PAGE and analyzed by Gelcode blue staining (Thermo Fisher Scientific) and immunoblot.

In vitro kinase experiments
Expression of 6xHis-Vac17 (97–355) and 6xHis-vac17-S222A (97–355) peptides in BL21 star DE3 cells was induced with 0.2 mM IPTG (Denville Scientific) at 16°C overnight. Cells were resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM Pefabloc [Sigma-Aldrich], and Complete EDTA-free protease inhibitor cocktail [Roche]) and lysed by sonication. Lysates were clarified via centrifugation at 20,000 g for 30 min at 4°C and incubated with Ni-NTA beads at 4°C with agitation. Immobilized HIS-tagged peptides were washed with 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, and 50 mM imidazole and then eluted in 50 mM sodium phosphate, pH 8.0, 300 mM NaCl, and 300 mM imidazole. Eluted peptides were dialyzed in 50 mM Tris, pH 8.0, and 50 mM NaCl overnight at 4°C. GST-Cla4 and GST-cla4-k594A were expressed in BL21 star DE3 cells via induction with 0.2 mM IPTG at 20°C for 3 h. Cells were resuspended in lysis buffer (50 mM sodium phosphate, pH 7.4, 125 mM NaCl, 10% glycerol, 2 mM MgCl2, 0.1% Tween-20, and 1 mM DTT) and then were washed with kinase buffer (50 mM Tris, pH 8.0, 1 mM EGTA, 2 mM MgCl2, and 1 mM DTT). GST fusion proteins were eluted in 500 µl kinase buffer containing 30 mM reduced glutathione. Recombinant proteins were then purified on a size exclusion column (HiLoad 16/60 Superdex 200; GE Healthcare). In vitro kinase assays were performed using kinase buffer containing 0.5 µg kinase, 2 µg substrate, 10 mM ATP, and 50 mM β-glycerophosphate in a volume of 50 µl and then incubated at 30°C for 1 h. Kinase reactions were terminated via the addition of sample buffer and then heated at 75°C for 10 min. Proteins were resolved via SDS-PAGE and analyzed by Gelcode blue staining (Thermo Fisher Scientific) and immunoblot.

α-factor cell synchronization
100 ml cells (OD, 0.2–0.4) were incubated in 2.5 µM α-factor (Zymo Research) for 2–3 h until 80% of cells were arrested in G1. Cells were washed in fresh media twice to remove α-factor. 1-ml aliquots were collected every 10 min for live-cell imaging, and images were taken within 5 min of collection.

Microscopy
To visualize vacuoles, cells were labeled with either (A) 12 µg FM4-64 in 250 µl media for 1 h and then washed twice and grown in 5 ml fresh media for one doubling time (2–3 h) or (B) 100 µM 7-aminochloromethylcoumarin (Thermo Fisher Scientific) for 30 min and then washed twice in fresh media. Live-cell images were obtained on a DeltaVision Restoration system (Applied Precision Ltd.) using an inverted epifluorescence microscope (IX-71; Olympus) with a charge-coupled device camera (CoolSNAP HQ; Photometrics) and processed in Photoshop (Adobe) and Fiji (ImageJ; National Institutes of Health).

Time-lapse microscopy
Glass-bottomed chambers (Lab-Tek II; Thermo Fisher Scientific) were treated overnight at 4°C with concanavalin A dissolved at 1 mg/ml in...
50 mM Hepes, pH 7.5, 20 mM calcium acetate, and 1 mM manganese sulfate and then washed with water and air dried for 30 min. Cells adsorbed to concanavalin A–treated chambers for 2 min. Unbound cells were removed by aspiration, and 250 μl fresh media was added. Time-lapse microscopy was performed at 2-min intervals using an inverted microscope (Ti-U; Nikon) with an iXon DV897 camera (Andor) with a 100x 1.4 NA oil immersion objective, a light engine (Lumencore), and a GFP/mCherry dual-band filter set (Chroma Technology Corp.).

Online supplemental material

Fig. S1 shows how deletion of Ste20 or Cla4 causes a partial defect in the termination of vacuole transport. Fig. S2 shows how Cla4 localizes to the vacuole after the vacuole contacts the bud cortex. Fig. S3 shows how Cla4 colocalizes with Vac17 and the vacuole at the bud cortex. Video 1 shows how Vac17-GFP disappears and the vacuole remains in the center of the cell. Video 2 shows how vac17-S222A-GFP and the vacuole persist at the bud cortex before moving to the mother-bud neck. Table S1 is a list of the yeast strains used in this study. Table S2 is a list of the plasmids used in this study.

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