

# Palmitoylation determines the function of Vac8 at the yeast vacuole

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Accepted 7 March 2006

*Journal of Cell Science* 119, 2477–2485 Published by The Company of Biologists 2006  
doi:10.1242/jcs.02972

## Summary

Palmitoylation stably anchors specific proteins to membranes, but may also have a direct effect on the function of a protein. The yeast protein Vac8 is required for efficient vacuole fusion, inheritance and cytosol-to-vacuole trafficking. It is anchored to vacuoles by an N-terminal myristoylation site and three palmitoylation sites, also known as the SH4 domain. Here, we address the role of Vac8 palmitoylation and show that the position and number of substrate cysteines within the SH4 domain determine the vacuole localization of Vac8: stable vacuole binding of Vac8 requires two cysteines within the N-terminus, regardless of the combination. Importantly, our data suggest that palmitoylation adds functionality to Vac8 beyond simple localization. A mutant Vac8 protein, in

which the palmitoylation sites were replaced by a stretch of basic residues, still localizes to vacuole membranes and functions in cytosol-to-vacuole transport, but can only complement the function of Vac8 in morphology and inheritance if it also contains a single cysteine within the SH4 domain. Our data suggest that palmitoylation is not a mere hydrophobic anchor required solely for localization, but influences the protein function(s).

Supplementary material available online at  
<http://jcs.biologists.org/cgi/content/full/119/12/2477/DC1>

Key words: Vac8, Palmitoylation, SH4 domain, Acylation, Src, Yeast vacuole

## Introduction

Proteins can be targeted and anchored to specific cellular membranes by covalently attached lipids (Roskoski, 2003). Palmitoylation is special among lipid modifications in that it is reversible: palmitate is attached to a target cysteine residue through a thioester linkage and can be removed by a thioesterase (Dietrich and Ungermann, 2004; Smotrys and Linder, 2004; Magee and Seabra, 2005). This reversibility suggests a regulatory role for palmitoylation, comparable with other post-translational modifications. It has been speculated that besides functioning as a membrane anchor, palmitate might induce conformational changes within the modified protein or recruit other proteins via lipid-protein interactions (Resh, 1999; Qanbar and Bouvier, 2003).

The hypothesis that palmitoylation is not only an anchor is supported by a number of observations. Various transmembrane proteins, such as G-protein-coupled receptors or SNAREs, are palmitoylated (el-Husseini Ael and Bredt, 2002; Bijlmakers and Marsh, 2003; Qanbar and Bouvier, 2003; Valdez-Taubas and Pelham, 2005). Palmitoylation of the GTPase-activating domain of RGS (regulator of G-protein signaling) family proteins increases their apparent activity (Osterhout et al., 2003). Certain multiply palmitoylated proteins require only a single palmitate for stable membrane association, indicating that the additional modifications serve additional functions (Martin and Busconi, 2000). Differences

in palmitoylation appear to delineate the functions of the three isoforms of the small GTPase Ras (H-, N- and K-Ras); in addition, recent evidence suggests that the extent of Ras palmitoylation accounts for its half-time on membranes (Goodwin et al., 2005; Rocks et al., 2005).

In this study, we asked whether the function of palmitoylation is restricted to targeting and anchoring a protein to the appropriate membrane or whether it encodes additional information. In addition, we questioned why certain proteins get multiply palmitoylated, although a single modification should be sufficient for anchoring. For this, we analyzed the vacuolar protein Vac8 from the yeast *Saccharomyces cerevisiae* (Fleckenstein et al., 1998; Pan and Goldfarb, 1998; Wang et al., 1998). Vac8 is an ideal model protein to study the functional consequences of palmitoylation, as it is multiply palmitoylated and has many functions. It is required for inheritance of vacuoles, homotypic vacuole fusion and cytosol-to-vacuole transport (CVT), an autophagy-like process (Wang et al., 1998; Scott et al., 2000; Veit et al., 2001). In contrast to vacuole inheritance and fusion, CVT does not require Vac8 palmitoylation (Wang et al., 1998; Scott et al., 2000).

Palmitoylation can occur at up to three sites in the N-terminal SH4 (Src homology 4) domain of Vac8 (Wang et al., 1998). SH4 domains are about 18 amino acids long and are characterized by a myristoylation motif (MGxxxS/Tx) and a

palmitoylation site (a cysteine residue) or several basic amino acids. Sequences homologous to the Vac8 SH4 domain are found in three other proteins: the rice protein kinase OSCP2 (Martin and Busconi, 2000), HASPB, an unconventionally secreted protein from *Leishmania major* (Denny et al., 2000), and AKAP18, which targets the cAMP-dependent protein kinase A to the plasma membrane (Fraser et al., 1998). Among these proteins, two features are conserved: the myristoylation motif, and a palmitoylated cysteine at position 4 or 5 that appears to be necessary and sufficient for protein activity (Fraser et al., 1998; Denny et al., 2000; Martin and Busconi, 2000). Vac8 is unique in that it has three potential palmitoylation sites. Here, we show that palmitoylation is

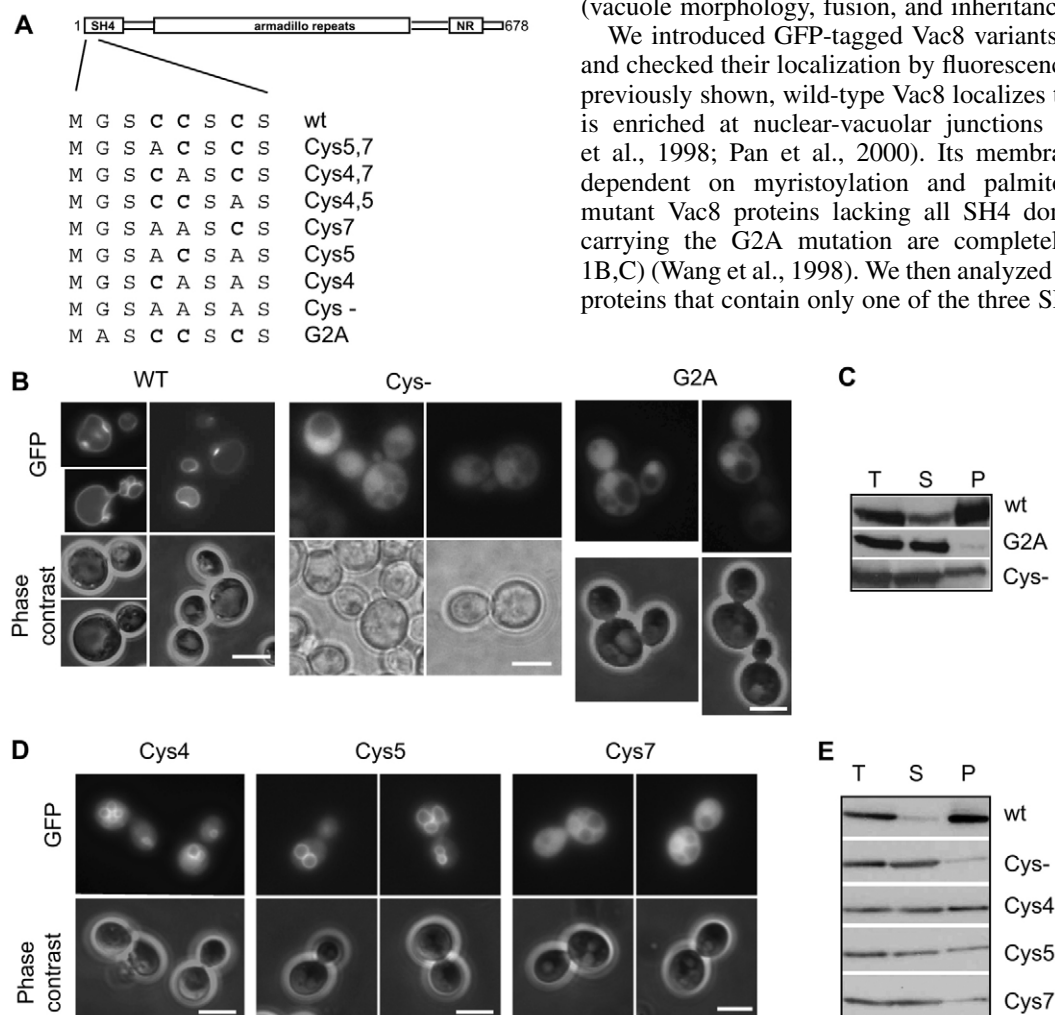
required for Vac8 function at the vacuole beyond membrane localization.

## Results

### Minimal palmitoylation requirements for Vac8 localization

Vac8 contains three potentially palmitoylated cysteines in its N-terminal SH4 domain (Fig. 1A). We asked whether these cysteines are redundant, or if they are differentially involved in the functional regulation of Vac8. To address this, we searched for the minimal palmitoylation requirement for stable localization of Vac8 by changing its cysteines individually or in combination, and then asked whether or not these minimal motifs support the palmitoylation-dependent functions (vacuole morphology, fusion, and inheritance).

We introduced GFP-tagged Vac8 variants into *vac8Δ* yeast and checked their localization by fluorescence microscopy. As previously shown, wild-type Vac8 localizes to the vacuole and is enriched at nuclear-vacuolar junctions (Fig. 1B) (Wang et al., 1998; Pan et al., 2000). Its membrane association is dependent on myristoylation and palmitoylation, because mutant Vac8 proteins lacking all SH4 domain cysteines or carrying the G2A mutation are completely cytosolic (Fig. 1B,C) (Wang et al., 1998). We then analyzed mutant Vac8-GFP proteins that contain only one of the three SH4 cysteines (Fig.



**Fig. 1.** SH4-domain alignments and localization of Vac8 mutants. (A) Domain structure of Vac8 and Vac8 mutants used in this study. Alanine mutations within the N-terminal region are indicated in bold. The top panel shows the domain structure of Vac8 (SH4, SH4 domain; NR, asparagine rich domain). Constructs were named according to their non-mutated cysteines. Numbers indicates the amino acid positions within the SH4 domain. (B) Localization of GFP fusion proteins in yeast. BJ3505 *vac8Δ* cells were transformed with plasmids expressing the indicated GFP fusion proteins. Their localization was visualized by fluorescence microscopy. Bars, 5 μm. (C) Subcellular localization of Vac8 mutants. Cells expressing the respective mutant proteins were lysed essentially as for vacuole purification. Lysates were cleared by low-speed centrifugation (300 g, 4°C) and half was saved (T), the other was fractionated by centrifugation (10 minutes, 13,000 g, 4°C) into pellet (P) and supernatant (S). Pellets and TCA-precipitated supernatant were analyzed by SDS-PAGE and western blotting with anti-Vac8. Fractionation was confirmed by control antibodies (see Fig. 4D). (D) In vivo localization of Vac8-GFP mutants with single cysteines. Fusion proteins encoding Vac8-Cys4-GFP, Vac8-Cys5-GFP or Vac8-Cys7-GFP were expressed in BJ3505 *vac8Δ* cells, and analyzed by fluorescence microscopy. Bars, 5 μm. (E) Subcellular localization. Cells with the indicated Vac8 cysteine mutations were fractionated and analyzed as described in Fig. 1C. Western blots were probed with antibodies to Vac8.

1D,E). According to a previous study, the presence of a cysteine near the N-terminal myristoylation site – regardless of its position – is sufficient to confer membrane binding (Navarro-Lerida et al., 2002). It was therefore surprising to us that the three cysteines do not contribute equally to its membrane localization. Although Vac8-GFP with either Cys4 or Cys5 shows a vacuolar localization, Vac8-Cys7-GFP is almost completely cytosolic (Fig. 1D,E), indicating that cysteine positioning in the SH4 domain is crucial for stable membrane binding. Subcellular fractionation studies confirmed these observations. The Cys7 and Cys<sup>-</sup> mutants are primarily cytosolic, whereas 40–50% of the Cys4 and Cys5 mutants were recovered in the pellet fraction (Fig. 1E). Wild-type Vac8 was found only in the pellet fraction, indicating that multiple cysteines enhance membrane binding. The same results were obtained regardless of the presence or absence of the GFP tag (see below). Thus, it is unlikely that the potential GFP dimerization has an influence on the behavior of our constructs.

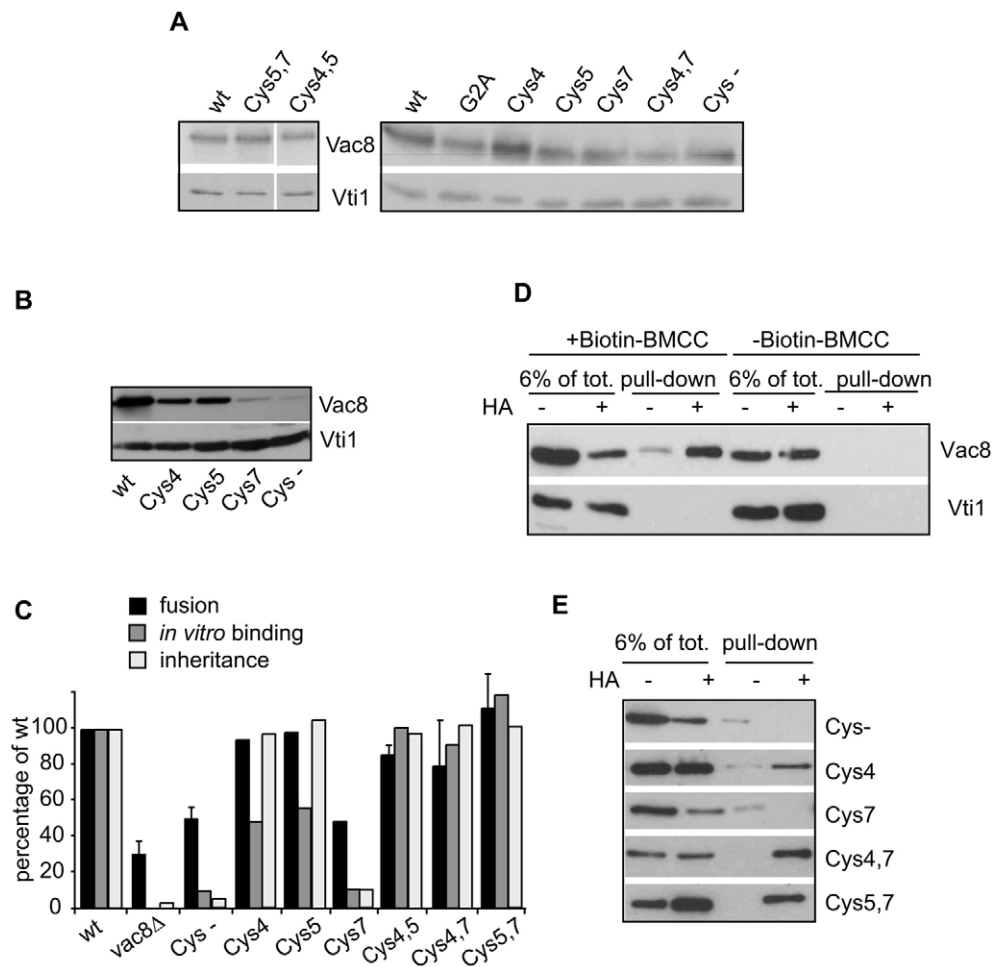
In order to extend these studies, we introduced constructs encoding the same mutations but lacking the GFP tag into

the genome of *vac8Δ* yeast, confirmed their comparable expression (Fig. 2A), isolated vacuoles from these mutant Vac8 strains and determined the membrane association of Vac8 (Fig. 2B,C, grey bars). As for the localization of GFP-tagged Vac8 (Fig. 1), about 40% of untagged Vac8 Cys4 and Cys5 mutants is present on membranes, whereas Vac8-Cys7 binding to membranes is as low as for the Cys<sup>-</sup> mutant (approximately 10%). We then asked whether any two cysteines within the SH4 domain could confer complete Vac8 localization. For this, we purified vacuoles from yeast strains with single Cys-to-Ala substitutions, and analyzed their Vac8 content. As shown in Fig. 2C, any combination of two cysteines confers a wild-type level of Vac8 attachment to vacuoles. It is therefore likely that palmitoylation of Cys7 occurs only if a proximal cysteine at position 4 or 5 has already been modified.

We then confirmed that membrane binding of our cysteine mutants was due to palmitoylation using a recently established assay, wherein the palmitate thioester linkage is cleaved by hydroxylamine, and the newly free cysteine is crosslinked to a biotin conjugate (Drisdell and Green, 2004; Valdez-Taubas and

**Fig. 2.** Analysis of membrane association and palmitoylation of Vac8 mutants. (A) Expression levels of endogenous Vac8 mutants. Proteins were extracted from BJ3505 strains expressing the respective mutants and analyzed by SDS-PAGE and western blotting with antibodies to Vac8 and Vti1. (B,C) Localization of Vac8 mutants to purified vacuoles. Vacuoles were isolated from strains carrying the indicated mutations in Vac8 as described in the Materials and Methods, then washed with 20 mM PIPES (pH 6.8), 150 mM KCl. Isolated vacuoles (30 μg) and total cell lysates were analyzed by SDS-PAGE and western blotting (B). (C) Effect of SH4 mutations in Vac8 on vacuole binding and inheritance. For vacuole binding, Vac8 bands in B were quantified by laser densitometry (grey bars). Fusion (black bars) was determined by the standard fusion assay (see Materials and Methods). Where indicated, error bars are standard deviations ( $n=3$ ). To determine vacuole inheritance (white bars), yeast cells of the respective mutants were analyzed by FM4-64 staining and fluorescence microscopy. For each strain at least 80 cells were counted. Inheritance of the wild-type control was on average 72%, which was set to 100% in the Figure.

(D) Palmitoylation of Vac8 detected by the biotin-switch method (Hou et al., 2005). Yeast cells were lysed, and free cysteines were quenched by NEM. The extract was then subjected to the biotin-switch procedure using hydroxylamine (HA) and Biotin-BMCC as a crosslinker. A fraction of the lysate (6%) was removed and proteins were TCA precipitated. The remaining modified proteins were captured by Neutravidin-agarose pull-down assay, and analyzed by SDS-PAGE and western blotting with the indicated antibodies. The wild-type control is the same as in Hou et al. (Hou et al., 2005). (E) Yeast cells expressing the respective Vac8 mutant analyzed as in D for palmitoylation.



Pelham, 2005). The biotinylated protein is then captured by Neutravidin agarose pull-down assay. Wild-type Vac8 was captured on Neutravidin beads if thioesters were first cleaved by hydroxylamine, rendering the cysteines accessible to the biotin crosslinker (Fig. 2D). Vac8 Cys<sup>-</sup> or the Vti1 SNARE were not recovered on Neutravidin beads after the same treatment, indicating that the modification is specific for the N-terminal SH4 domain. We then subjected all our mutant Vac8 proteins to the same treatment. Vac8 constructs that showed complete or partial localization were pulled down on Neutravidin agarose, whereas the poorly membrane-associated Cys7 mutant was not (Fig. 2E), indicating that their localization correlates with palmitoylation.

### Effect of SH4 mutations on Vac8 function

In order to determine whether palmitoylation/localization of the Vac8 SH4 domain mutants correlates to function, we assessed their ability to support vacuole fusion and inheritance. We found that their function in both processes correlates to vacuole binding (Fig. 2C): Vac8-Cys7, which localizes poorly to vacuoles, fused and inherited as poorly as the Cys<sup>-</sup> mutant, whereas partial localization of Vac8 (conferred by Cys4 or Cys5) was sufficient for wild-type-like fusion and inheritance. Thus, the ability of cysteines within the Vac8 SH4 domain to drive membrane association is clearly correlated with functionality. Interestingly, it appears that membrane binding is more important for vacuole inheritance than for fusion (see Cys7 and Cys<sup>-</sup>, Fig. 2C).

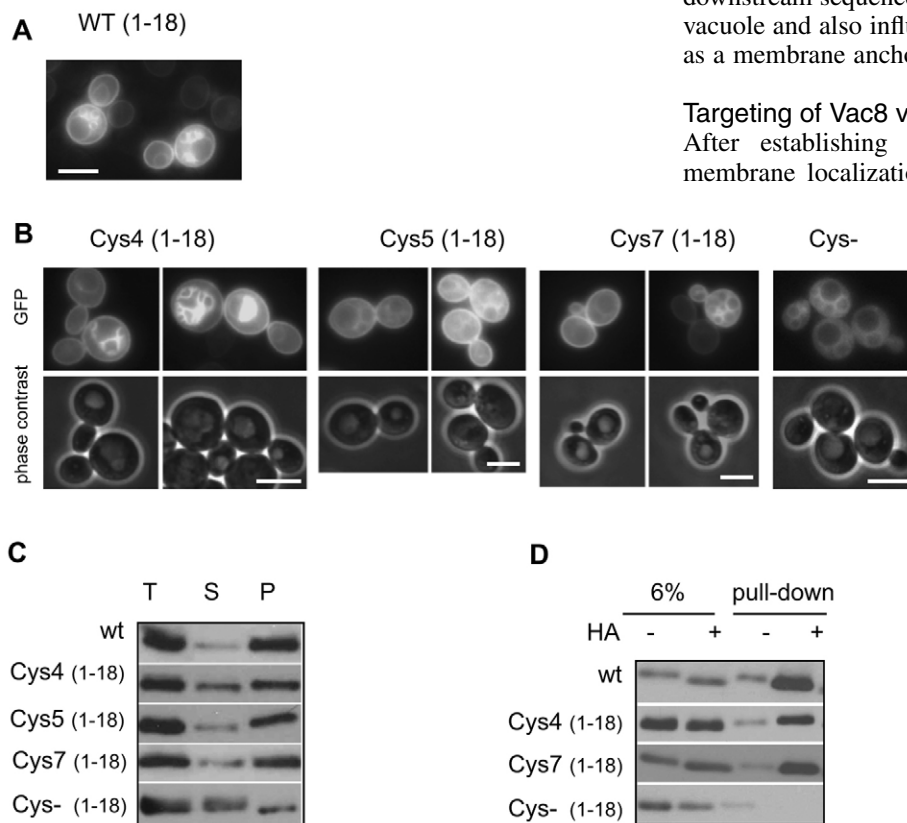
### Localization of GFP by a minimal SH4 motif

Our data indicate that efficient vacuole localization of Vac8

requires two cysteines within the SH4 domain. We asked whether the Vac8 SH4 motif alone contains sufficient information to target a heterologous protein to the vacuole. When fusion proteins of the Vac8 SH4 domain and GFP were expressed in yeast, we observed targeting of the fusion proteins to intracellular membranes and the plasma membrane (Fig. 3A), similar to observations made in mammalian cells (McCabe and Berthiaume, 2001). Does efficient membrane targeting of GFP, like Vac8, also require two cysteines? To answer this, we generated strains with mutant SH4-GFP chimeras [Vac8(1-18)-GFP in the *vac8Δ* background], which contain only Cys4, Cys5 or Cys7. Interestingly, any one cysteine is sufficient to stably bind SH4-GFP to intracellular membranes and the plasma membrane as shown both by fluorescence microscopy (Fig. 3B) and subcellular fractionation (Fig. 3C); we confirmed that these mutants are palmitoylated in vivo (Fig. 3D). A chimera lacking all three N-terminal cysteines was bound poorly to internal membranes and was not found at the plasma membrane (Fig. 3B,C); in contrast to Vac8-GFP, myristoylation of the SH4 domain seems to be sufficient for mediating weak membrane binding, which could be due to the GFP dimerization. Thus, in contrast to the full-length Vac8 protein, palmitoylation of the SH4 domain alone appears to be independent of the cysteine location, which is in agreement with previous data (Navarro-Lerida et al., 2002). Moreover, the SH4 domain requires only a single cysteine to target GFP, whereas in the context of Vac8 multiple cysteines are required for wild-type-like localization. We conclude that the SH4 domain is a universal membrane-targeting motif that is recognized and palmitoylated at membranes and requires downstream signals for the localization to a specific membrane. In the case of Vac8, the downstream sequence is required to direct it exclusively to the vacuole and also influences the robustness of the SH4 domain as a membrane anchor.

### Targeting of Vac8 via a polybasic N-terminal domain

After establishing a correlation between palmitoylation, membrane localization and functionality of Vac8, we asked



**Fig. 3.** Targeting of GFP by Vac8 wild-type and mutant SH4 domains in yeast. GFP-fusion proteins encoding the first 18 amino acids of Vac8 (A) or the indicated mutations (B) were expressed in BJ3505 *vac8Δ* cells and analyzed by fluorescence microscopy. Bars, 5  $\mu$ m. (C) Subcellular localization of Vac8(1-18)-GFP. The indicated cells were analyzed as in Fig. 1C. Proteins were analyzed by SDS-PAGE and western blotting with anti-GFP antibodies. (D) Palmitoylation of the SH4 constructs. The biotin-switch method was used as described in Fig. 2D and Hou et al. (Hou et al., 2005). Western blots were decorated as in C.

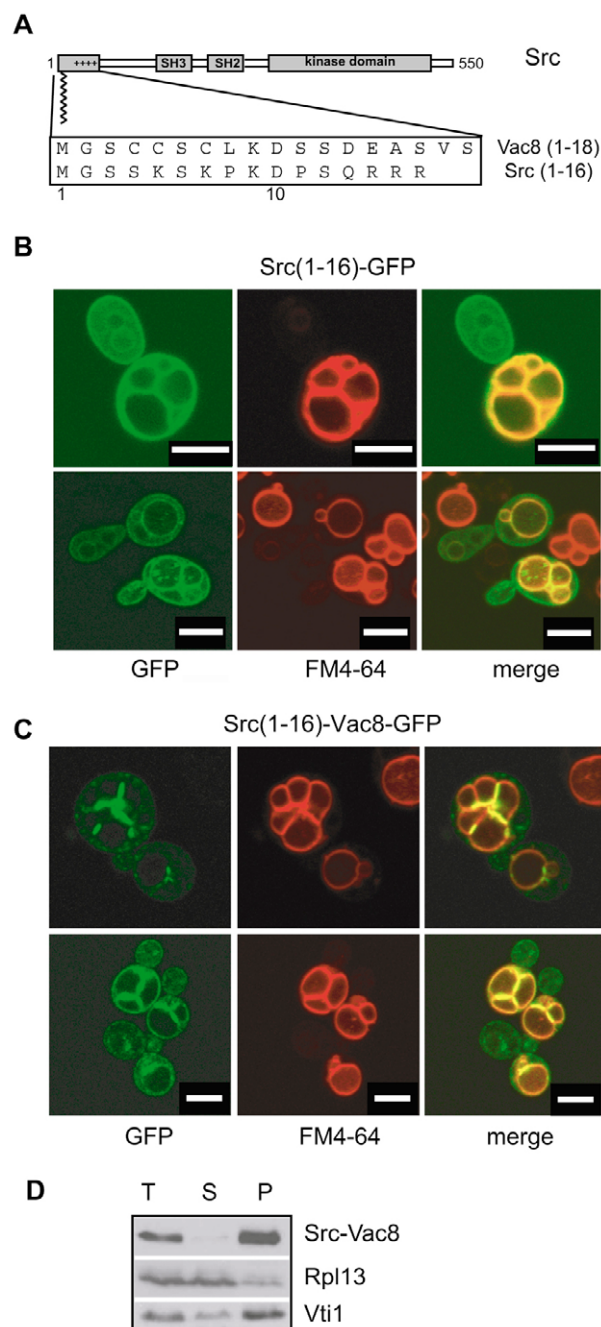


whether Vac8 function depends on its mere membrane attachment or on the palmitate modification per se. We therefore sought to target Vac8 to membranes in a palmitoylation-independent manner. For this we replaced its SH4 domain (myristate/palmitate) with the SH4 domain (myristate/basic stretch) of the mammalian Src tyrosine kinase, which is primarily targeted to the plasma membrane (Fig. 4A). In mammalian cells, both SH4 domains target GFP to the plasma membrane and endosomes (McCabe and Berthiaume, 2001). Similarly, our Src(1-16)-GFP fusion protein localized like Vac8(1-18)-GFP, staining the plasma membrane and multilobed vacuoles (Fig. 4B). Having established that the Src SH4 domain can act as a membrane anchor in yeast, we replaced the N-terminal 18 amino acids of Vac8 with the Src(1-16) sequence, and expressed the chimeric Src-Vac8 protein with a C-terminal GFP tag in the *vac8* deletion background. Src-Vac8-GFP was not found on the plasma membrane, but almost exclusively localized to the vacuole, like wild-type Vac8-GFP (Fig. 4C). We confirmed its localization by subcellular fractionation (Fig. 4D). These data indicate that as long as Vac8 has a functional SH4 domain, it is its remaining sequence that restricts its localization to vacuoles.

#### Vac8 function depends on palmitoylation

Wild-type cells contain one to three large vacuoles (Fig. 1B) and a defect in vacuole fusion often results in fragmentation of vacuoles in vivo. Therefore, the multilobed vacuole morphology in the Src-Vac8-GFP strain (Fig. 4C) was a first indication that it cannot complement the absence of wild-type Vac8, despite its appropriate vacuolar localization. In order to directly assess the functionality of the Src-Vac8 chimera, we tested its ability to support in vitro vacuole fusion and in vivo vacuole inheritance. The former assay monitors content mixing between isolated vacuoles (see Materials and Methods). We observed that, compared with vacuoles with wild-type Vac8, Src-Vac8 vacuoles were deficient in vacuole fusion (Fig. 5A). Fusion was comparable to a Vac8 mutant lacking the N-terminal three cysteines (Cys<sup>-</sup>). To test for mother-to-daughter inheritance of Src-Vac8-bearing vacuoles, we stained yeast vacuoles in intact cells with the lipophilic dye FM4-64 and then followed them by fluorescence microscopy (Catlett and Weisman, 1998). The lack of a labeled vacuole in the bud indicates defective inheritance. Whereas wild-type Vac8 supports inheritance of vacuoles into the daughter cell, Src-Vac8 does not, and similar defects were observed for the *vac8Δ* and Cys<sup>-</sup> mutants (Fig. 5B,C) (Wang et al., 1998). These data demonstrate that although the Src SH4 domain is sufficient to localize Vac8 to vacuoles, it does not functionally replace the Vac8 SH4 domain. Importantly, Src-Vac8 is functional in a third Vac8-dependent reaction, the CVT pathway of aminopeptidase I (Ape1), an autophagy-like process in yeast that does not depend on Vac8 palmitoylation. As shown in Fig. 5D, Src-Vac8 promotes Ape1 maturation (unlike cells lacking Vac8), which indicates that the Src-SH4 domain does not impair Vac8 function per se. From this, we conclude that the cysteine-containing SH4 domain of Vac8 is not only required for localization of the protein, but also determines its function in fusion and inheritance.

One possible reason for the inability of Src-Vac8 to complement the palmitoylation-dependent functions of Vac8 could be its physical interaction with lipids. Hydrophobic

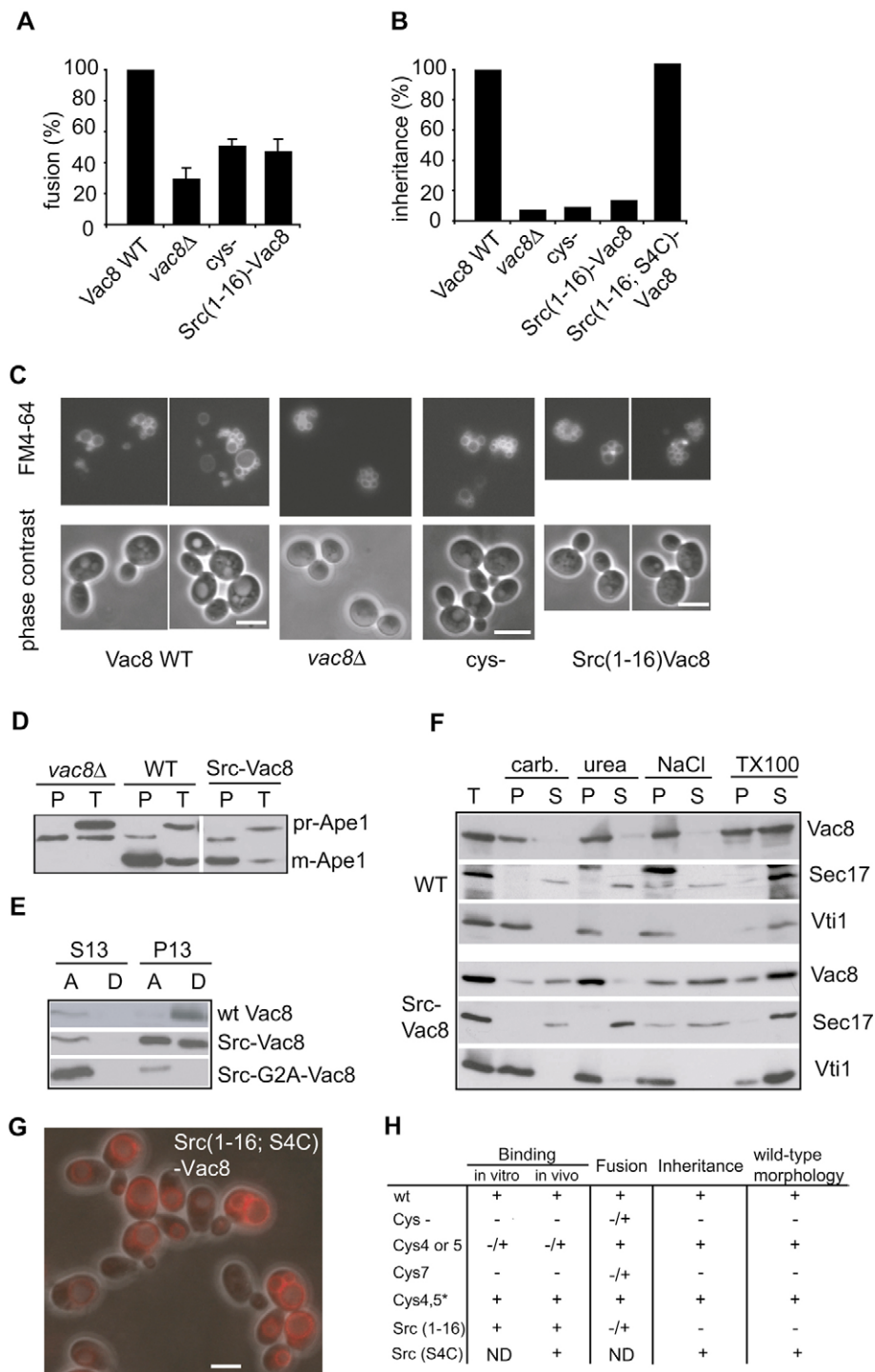


**Fig. 4.** Vacuole targeting of Vac8 through the Src kinase SH4 domain. (A) Comparison of the SH4 sequence of Vac8 and the Src kinase. Alignment of the two SH4 domains is shown. (B) Localization of Src(1-16)-GFP by confocal fluorescence microscopy. The GFP-fusion protein was expressed from a plasmid in the *vac8Δ* background. Vacuoles were visualized with the lipophilic dye FM4-64. (C) Vacuole targeting of Vac8 through the Src kinase SH4 domain. Src-Vac8-GFP protein was expressed from a plasmid in *vac8Δ* cells and monitored by confocal microscopy in FM4-64 stained cells. Bars, 5 μm. (D) Subcellular localization of Src-Vac8. Fractionation was done as in Fig. 1C. Pellets and TCA-precipitated supernatant were analyzed by SDS-PAGE and western blotting with anti-Vac8, anti-Rpl13 and anti-Vti1 antibodies. Rpl13 is a ribosomal subunit (cytosolic marker), Vti1 is found on vacuoles (membrane marker). See Materials and Methods for details.

partitioning is a simple test to analyze the hydrophobicity of a protein. For this, organelle membranes are detergent solubilized in Triton X-114 at 4°C. Upon warming, the detergent extract partitions into an aqueous and a detergent phase, which can be separated by centrifugation. Hydrophobic proteins are found in the detergent phase, whereas more hydrophilic proteins appear in the aqueous phase. When membranes from wild-type and Src-Vac8 strains were subjected to hydrophobic partitioning we identified clear differences between the Vac8 proteins (Fig. 5E). Whereas wild-

type Vac8 was present only in the detergent phase, at least half of the Src-Vac8 protein was also found in the aqueous phase. However, membrane binding of Src-Vac8 still required a myristoyl anchor, because a G2A mutant of Src-Vac8 was primarily cytosolic (Fig. 5E). In addition, some Src-Vac8 is removed from membranes by carbonate or salt, but not urea, whereas the wild-type protein remained associated with membranes (Fig. 5F). Thus, even though Src-Vac8 localizes exclusively to vacuoles, it appears to be less hydrophobic and binds less tightly to membranes.

**Fig. 5.** Vac8 function depends on palmitoylation. (A) Vacuole fusion. Vacuoles were isolated from the two *vac8Δ* tester strains carrying no Vac8 protein (*vac8Δ*), Vac8 wild-type, the Cys<sup>-</sup> mutant (*cys*<sup>-</sup>) or the Src-Vac8 chimera, and incubated in a standard vacuole fusion reaction for 90 min at 26°C (see Materials and Methods), and then assayed for alkaline phosphatase activity. The average of four independent experiments is shown; error bars are standard deviations. (B) Vacuole inheritance. Analysis of yeast cells carrying the respective mutants was done as in Fig. 2C. (C) Morphology and inheritance of the mutant strains. Inheritance of FM4-64 labeled vacuoles was visualized by fluorescence microscopy. Bars, 5 μm. (D) AP-1 maturation assay. Cells from the indicated strains were fractionated into total (T) and a membrane containing pellet (P) fraction. Proteins were analyzed by SDS-PAGE and western blots were probed with antibodies to Ape1. pr, immature protein; m, mature protein. (E) Triton X-114 partitioning of wild-type Vac8 and Src-Vac8. Cells expressing the indicated Vac8 variant were fractionated into a P13 and S13 fraction, and subjected to Triton X-114 partitioning. Proteins in the aqueous (A) and detergent (D) phase were TCA precipitated and analyzed by SDS-PAGE and western blotting with antibodies to Vac8. (F) Membrane association of Vac8 and Src-Vac8. Vacuoles (30 μg) from strains expressing the respective Vac8 variants were subjected to addition of 100 mM Na<sub>2</sub>CO<sub>3</sub> (carb.), 6 M urea, 1 M NaCl or 1% Triton X-100. Samples were vortexed, incubated on ice for 30 minutes, and then centrifuged (30 minutes, 100,000 g, 4°C) (Ungermann and Wickner, 1998). Pellet and TCA-precipitated supernatant were dissolved in SDS sample buffer, and analyzed by SDS-PAGE and western blotting with the indicated antibodies. The same wild-type control has also been used in Hou et al. (Hou et al., 2005). (G) Morphology and Inheritance of Src (1-16; S4C)-Vac8. Analysis was done as in Fig. 2C. Phase-contrast and FM4-64 images were superimposed using Photoshop 7.0. Bar, 5 μm. (H) Summary of data from this study. The asterisk on Cys4,5 indicates that similar results were obtained for all single Cys-Ala mutations; ND, not determined.



Because the palmitoylation machinery on yeast vacuoles can recognize the Src SH4 domain (Hou et al., 2005), we tested whether the introduction of a single cysteine at position 4 into the Src SH4 domain would rescue the phenotype of the Src-Vac8 mutant. The Src(1-16; S4C)-Vac8 fusion protein was expressed at wild-type levels and found on vacuoles (not shown), and indeed rescued inheritance and morphology (Fig. 5B,G). Our data therefore suggest that palmitoylation of the SH4 domain is indeed a key determinant of Vac8 function at the vacuole.

## Discussion

Our study shows that Vac8 function depends on palmitoylation. Vac8 must be multiply palmitoylated to anchor stably to vacuoles and fulfill its function in vacuole fusion and inheritance (summarized in Fig. 5H). Targeting depends on the SH4 domain, which is a minimal membrane-targeting motif in yeast and mammalian cells. Replacement of the Vac8 SH4 domain (myristate/palmitate) with that of Src (myristate/basic stretch) is sufficient to localize Vac8 to vacuoles and allows for Vac8 function in the CVT pathway, but rendered the protein non-functional in terms of its involvement in vacuole fusion and inheritance. Importantly, introducing a single cysteine into the Src sequence rescued the vacuole morphology and inheritance defect. We conclude that palmitoylation is not only important for targeting Vac8 to the vacuole, but that it affects its function.

How could palmitoylation affect Vac8 function? For each of its functions, Vac8 interacts with specific proteins (Pan and Goldfarb, 1998; Wang et al., 1998; Scott et al., 2000; Veit et

al., 2001; Ishikawa et al., 2003). Therefore, palmitoylation of Vac8 could directly affect these interactions, either by forming direct lipid-protein interactions or by inducing conformational changes that permit certain protein-protein interactions. Another possibility is that palmitoylation directs Vac8 to special domains on the vacuole membrane. Such domains may exist on vacuoles, because lipid-enriched domains form during and are required for vacuole fusion (Fratti et al., 2004; Kato and Wickner, 2001). A requirement for such regulatory mechanisms might explain the seemingly contradictory phenotypes observed with Src-Vac8. Although the protein is properly localized and functional in the CVT pathway (Fig. 5), its altered SH4 domain might interfere with the interaction of Vac8 with other proteins or the lipid bilayer. Indeed, upon Triton X-114 partitioning, membrane-bound Src-Vac8 was found in both the aqueous and detergent phases (Fig. 5E) and was more readily extracted from membranes than wild-type Vac8 (Fig. 5F), indicating that it interacts more weakly with the lipid bilayer than Vac8. Based on these observations we speculate that the partitioning of Src-Vac8 into the aqueous phase could reflect the ability of the Vac8 moiety to accommodate the hydrophobic myristoyl tail within the protein. Such a switch-like mechanism has been discussed for other myristoylated proteins like HIV-Gag (Resh, 2004).

Previous studies showed that Vac8 becomes palmitoylated early in the fusion reaction, and that this is necessary for efficient fusion (Veit et al., 2001; Wang et al., 2001). Acylation of Vac8 requires a novel function of the SNARE Ykt6 and occurs in an ATP-independent, temporally-controlled subreaction on vacuoles (Dietrich et al., 2004; Dietrich et al.,

**Table 1. *S. cerevisiae* strains used in this study**

Strain	Genotype	Reference
CUY72	<i>MATa pep4Δ::HIS3 prb1-Δ1.6R HIS3 lys2-208 trp1-Δ101 ura3-52 gal2 can vac8Δ::TRP1</i>	Veit et al., 2001
CUY73	<i>MATa leu2-3 leu2-112 ura3-52 his3-Δ200 trp1-Δ101 lys2-801 suc2-Δ9 pho8:: TRP1 vac8Δ::HIS3</i>	Veit et al., 2001
CUY363a	CUY72; <i>VAC8pr::pRS406-VAC8pr-VAC8</i>	This study
CUY363b	CUY73; <i>VAC8pr::pRS406-VAC8pr-VAC8</i>	This study
CUY861a	CUY72; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys4</sup></i>	This study
CUY861b	CUY73; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys4</sup></i>	This study
CUY367a	CUY72; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys5</sup></i>	This study
CUY367b	CUY73; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys5</sup></i>	This study
CUY366a	CUY72; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys7</sup></i>	This study
CUY366b	CUY73; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys7</sup></i>	This study
CUY352a	CUY72; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys4,5</sup></i>	This study
CUY352b	CUY73; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys4,5</sup></i>	This study
CUY365a	CUY72; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys4,7</sup></i>	This study
CUY365b	CUY73; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys4,7</sup></i>	This study
CUY364a	CUY72; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys5,7</sup></i>	This study
CUY364b	CUY73; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys5,7</sup></i>	This study
CUY369a	CUY72; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys-</sup></i>	This study
CUY369b	CUY73; <i>VAC8pr::pRS406-VAC8pr-Vac8<sup>Cys-</sup></i>	This study
CUY1092a	CUY72; <i>VAC8pr::pRS406-VAC8pr-Src<sup>1-16</sup>-Vac8<sup>Δ1-18</sup></i>	This study
CUY1092b	CUY73; <i>VAC8pr::pRS406-VAC8pr-Src<sup>1-16</sup>-Vac8<sup>Δ1-18</sup></i>	This study
CUY1615	CUY72; <i>VAC8pr::pRS406-VAC8pr-Src<sup>1-16(G2A)</sup>-Vac8<sup>Δ1-18</sup></i>	This study
CUY940	CUY72; <i>pRS426- NOP1pr-VAC8-GFP</i>	This study
CUY973	CUY72; <i>pRS426- NOP1pr-Vac8<sup>Cys4</sup>-GFP</i>	This study
CUY1042	CUY72; <i>pRS426- NOP1pr-Vac8<sup>Cys5</sup>-GFP</i>	This study
CUY1063	CUY72; <i>pRS426- NOP1pr-Vac8<sup>Cys7</sup>-GFP</i>	This study
CUY948	CUY72; <i>pRS426- NOP1pr-Vac8<sup>Cys-</sup>-GFP</i>	This study
CUY1549	CUY72; <i>pRS426- NOP1pr-Vac8<sup>G2A</sup>-GFP</i>	This study
CUY987	CUY72; <i>pRS426- NOP1pr-Src<sup>1-16</sup>-GFP</i>	This study
CUY942	CUY72; <i>pRS426- NOP1pr-Vac8<sup>Δ1-18</sup>-GFP</i>	This study
CUY1006	CUY72; <i>pRS426- NOP1pr-Src<sup>1-16</sup>-Vac8<sup>Δ1-18</sup>-GFP</i>	This study
CUY1229	CUY73; <i>pRS426- NOP1pr-Vac8<sup>Δ1-18 Cys5</sup>-GFP</i>	This study
CUY1230	CUY73; <i>pRS426- NOP1pr-Vac8<sup>Δ1-18 Cys7</sup>-GFP</i>	This study
CUY1909	CUY73; <i>VAC8pr::pRS406-VAC8pr- Src<sup>1-16; S4C</sup>-Vac8<sup>Δ1-18</sup></i>	This study



2005). Potentially, only one cysteine of Vac8 is initially palmitoylated when we isolate vacuoles. Our *in vitro* palmitoylation assay with purified vacuoles may then detect Vac8 palmitoylation at one or two additional cysteines (Veit et al., 2001; Dietrich et al., 2004), which would be required for stable association and vacuole fusion (Veit et al., 2001; Wang et al., 2001). The vacuolar DHHC protein Pfa3 has been identified as a factor required for Vac8 localization (Hou et al., 2005; Smotrys et al., 2005). In the absence of Pfa3 some Vac8 is still localized to vacuoles, whereas the Cys4 mutant is completely cytosolic (our unpublished results). A further understanding of Vac8 palmitoylation will therefore require a detailed analysis of the vacuolar palmitoylation machinery.

## Materials and Methods

### Materials

All biochemical reagents were purchased from Sigma (Steinheim, Germany) or Roth (Karlsruhe, Germany), unless indicated.

### Yeast strains

*Saccharomyces cerevisiae* strains are listed in Table 1. Cells that hosted plasmids were cultured in SDC-Ura medium; all other cells were grown in YPD medium.

### Generation of integrative plasmids expressing Vac8

For VAC8pr, 800 nucleotides upstream of the VAC8 start codon were PCR amplified from yeast genomic DNA and ligated into *HindIII/EcoRI* digested pRS406 to generate pRS406-VAC8pr. VAC8 and the various vac8 mutants were amplified using the primers listed in Table S1 in supplementary material, the 5' primer sequences encoded wild-type or modified N-terminal amino acids. All fragments were *EcoRI/BamHI* inserted into pRS406-VAC8pr. Plasmids were cut with *XmaI* (a unique restriction site within the VAC8 promoter region) and inserted into the VAC8 promoter of BJ3505 and DKY6281 strains lacking the VAC8 open reading frame (CUY72 and CUY73). Expression of mutant proteins was confirmed by western blotting.

### Generation of 2 $\mu$ plasmids expressing Vac8-GFP

GFP was amplified from the plasmid pGL (generous gift from Sean Munro, Cambridge, UK). Further mutations in Vac8(1-18) were introduced into the 5' primer (Table S2 in supplementary material), and amplified using pRS426-NOP1pr-Vac8<sup>1-16</sup>-GFP as template. All plasmids were transformed into CUY72.

### Microscopy

For FM4-64 staining, yeast cells were grown in YPD to OD<sub>600</sub> <0.5. A 250  $\mu$ l aliquot was removed and incubated with 50  $\mu$ M FM4-64 for 1 hour (pulse). Cells were washed and resuspended in 5 ml YPD, grown for 3 hours (chase), and then analyzed by fluorescence microscopy as described (LaGrassa and Ungermann, 2005). Confocal fluorescence microscopy was done using a LSM 510 Meta from Carl Zeiss, Jena, Germany.

### Vacuole purification and fusion assay

Vacuoles were purified as previously described (Haas, 1995). Fusion reactions (30  $\mu$ l) containing 3  $\mu$ g of each vacuole type were incubated at 26°C in reaction buffer (125 mM KCl, 5 mM MgCl<sub>2</sub>, 20 mM PIPES/KOH, pH 6.8, 200 mM sorbitol), a protease inhibitor cocktail, 10  $\mu$ M Coenzyme A, and an ATP-regenerating system. One unit of fusion activity corresponds to 1  $\mu$ mol *p*-nitrophenol phosphate hydrolyzed per minute per  $\mu$ g pep4 $\Delta$  vacuoles at 30°C.

### Subcellular fractionation

Cultures (20 ml) were grown to OD<sub>600</sub>=0.8-1.0 in YPD. Until lysis, cells were treated exactly as for the vacuole purification. Pelleted spheroplasts were resuspended in 1 ml lysis buffer consisting of 200 mM sorbitol, 50 mM KOAc, 2 mM EDTA, 20 mM HEPES-KOH pH 6.8, 1 $\times$  protease inhibitor cocktail (Haas, 1995), 1 mM PMSF and 1 mM DTT. Cells were then osmotically lysed in 2  $\mu$ g/ml DEAE-dextran. The total extract was centrifuged at 400 *g* in a microfuge for 5 minutes at 4°C and the supernatant was then centrifuged for 15 minutes at 13,000 *g*, and pellet (P13; this fraction contains vacuoles) and supernatant (S13) fractions were collected.

### Triton X-114 partitioning

Yeast cells (20 OD<sub>600</sub>) were separated into P13 and S13 fractions. Then, 500  $\mu$ l of each fraction were combined with 500  $\mu$ l of phosphate-buffered saline (PBS) containing 2% Triton X-114 with protease inhibitors (1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide and 10  $\mu$ g pepstatin A). The

samples were incubated for 20 minutes at 4°C to solubilize membrane proteins. To separate the detergent-enriched and aqueous phases, samples were incubated for 3 minutes at 30°C, followed by centrifugation (10 minutes, 20,000 *g*, room temperature). The detergent phase was washed twice with PBS containing 0.05% Triton X-114 and the aqueous phase with 2% Triton X-114. Each phase was transferred into a new tube, proteins were precipitated with 13% trichloroacetic acid, and analyzed by SDS-PAGE and western blotting.

### Biotinylation assay

Palmitoylation was detected by the biotin-switch approach as described (Hou et al., 2005).

We thank Daniel Klionsky, Michael Knop, Sean Munro, and Herbert Tschochner for plasmids and antibodies, members of the Ungermann lab for discussions, and Nadine Decker and Gabriela Müller for expert technical assistance. This work was supported by a grant from the DFG (UN111/2-3), the SFB 638, the EMBO Young Investigator program, the Fonds der Chemischen Industrie and by predoctoral fellowships of the Boehringer Ingelheim Fonds (to L.E.P.D., K.S. and H.H.) and the National Science Foundation Graduate Research Fellowship Program (to T.J.L.).

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