

The DHHC protein Pfa3 affects vacuole-associated palmitoylation of the fusion factor Vac8

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Vacuole biogenesis depends on specific targeting and retention of peripheral membrane proteins. At least three palmitoylated proteins are found exclusively on yeast vacuoles: the fusion factor Vac8, the kinase Yck3, and a novel adaptor protein implicated in microautophagy, Meh1. Here, we analyze the role that putative acyltransferases of the DHHC family play in their localization and function. We find that Pfa3/Ynl326c is required for efficient localization of Vac8 to vacuoles *in vivo*, while Yck3 or Meh1 localization is not impaired in any of the seven DHHC deletions. Vacuole-associated Vac8 appears to be palmitoylated in a *pfa3* mutant, but this population is refractive to further palmitoylation on isolated vacuoles. Vacuole morphology and inheritance, which both depend on Vac8 palmitoylation, appear normal, although there is a reduction in vacuole fusion. Interestingly, Pfa3 is required for the vacuolar localization of not only an SH4 domain that is targeted by myristate/palmitate (as in Vac8) but also one that is targeted by a myristate/basic stretch (as in Src). Our data indicate that Pfa3 has an important but not exclusive function for Vac8 localization to the vacuole.

Yck3 | SH4 domain | acylation | membrane targeting

Protein and lipid trafficking along the endomembrane system occurs by vesicular transport (1). Of all proteins implicated in vesicle fusion and fission, only a subset is permanently associated with membranes via transmembrane segments, whereas most are recruited to the membrane from the cytoplasm. The latter proteins depend on membrane receptors, lipids, or lipid anchors for binding to their appropriate target membrane (2). This poses the question of how the recruitment of these proteins is coordinated with their function.

Palmitoylation has been discussed as a special lipid modification. It may direct proteins to specific membrane domains (3–5), and it is the only common lipid modification that is reversible, permitting cycling of a protein between membranes and cytosol. The identification and characterization of the underlying acylation/deacylation machinery is therefore critical for understanding the function of palmitoylation. Recently, biochemical and genetic analyses have identified several proteins that are required for palmitoylation, including those of the so-called DHHC-CRD family of polytopic membrane proteins (6). Yeast contains seven homologs (Erf2, Swf1, Yol003c, Akr1, Akr2, Ydr459c, and Ynl326c/Pfa3), which seem to be distributed throughout the endomembrane system as suggested by the GFP database and recent studies. At the ER, Swf1 is required for palmitoylation of Tlg1 (7), and Erf2 promotes Ras2 palmitoylation (8, 9). The Golgi-localized Akr1 is responsible for palmitoylation of the casein kinase I (CKI) isoform Yck2 (10). Similarly, several of the 20 or so mammalian DHHC proteins localize to distinct organelles, with critical roles for the palmitoylation of PSD-95, Ras, and SNAP-25 (11–13). This wide distribution of proteins involved in palmitoylation suggests that palmitoylation is not restricted to one organelle but may occur at multiple sites.

We are interested in protein palmitoylation at the yeast vacuole. The fusion factor Vac8 is myristoylated at the N-

terminal glycine and palmitoylated at up to three N-terminal cysteines (called SH4 domain). Palmitoylation is observed during the *in vitro* fusion of purified vacuoles and is required for Vac8's function in fusion and inheritance (14) (K.S., L.E.P.D., H.H., T.J.L., and C.U., unpublished work). Another regulator of vacuole fusion, the CKI isoform Yck3, is tail-anchored to vacuoles through palmitoylation of a cysteine string-like motif (15, 16). Recently, a third palmitoylated vacuolar protein, Ego1/Meh1, was identified as a factor involved in microautophagy (refs. 17 and 18 and our unpublished observations). Whereas most of Yck3 seems to be palmitoylated before its sorting to the vacuole (15), a large proportion of Vac8 can be palmitoylated on vacuoles. We have previously shown that the SNARE Ykt6 is involved in palmitoylation of vacuole-associated Vac8. Antibodies to Ykt6 block Vac8 palmitoylation, and purified Ykt6 can support *in vitro* palmitoylation of Vac8 when present in stoichiometric amounts (19). To search for additional factors involved in palmitoylation of vacuolar proteins, we determined whether any of the seven DHHC proteins are required for the localization and palmitoylation of Vac8, Yck3, or Meh1/Ego1. Here, we present evidence that the vacuolar DHHC protein Ynl326c contributes to the localization of Vac8 and affects palmitoylation, but not localization, of Yck3. No single DHHC protein could be identified that is responsible for Yck3 or Meh1/Ego3 localization. Importantly, Ynl326c deletion does not completely abolish Vac8's sorting, palmitoylation, and function, indicating that its membrane association involves multiple factors, including two factors that affect its palmitoylation.

Methods

Yeast Strains and Molecular Biology. BY4741 yeast strains (MATa his3 Δ leu2 Δ met15 Δ ura3 Δ) with deletions in the DHHC genes (*erf2 Δ* , *akr1 Δ* , *akr2 Δ* , *ydr459c Δ* , *yol003c Δ* , *swf1 Δ* , *pfa3 Δ*) were purchased from EUROSCARF (Frankfurt). Deletions in *pfa3* in BJ3505 (MATa pep4::HIS3 prb1- Δ 1.6R HIS3 lys2–208 trp1 Δ 101 ura3–52 gal2 can) and DKY6281 (MATa leu2–3 leu2–112 ura3–52 his3- Δ 200 trp1- Δ 101 lys2–801 suc2- Δ 9 pho8::TRP1) were generated by transforming PCR products that introduce a *URA3* (BJ) or a *HIS3* (DKY) marker into the *PF3* locus. Loss of the gene was confirmed by PCR. To facilitate screening for Yck3, a pRS416-NOP1pr-GFP-YCK3 plasmid (16) was introduced into the BY4741 deletion strains. Vac8 was tagged genomically at the C terminus with GFP by using pYM29 (20)

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as a template for PCR, followed by selection on yeast extract/peptone/dextrose (YPD) plus geneticin. Pfa3 was tagged with the TAP tag by using a similar approach and the same selection procedure. N-terminal tagging of Yck3 with GFP in BJ3505 was done by PCR amplifying a TRP1-PHO5pr-GFP-Myc cassette that replaced the endogenous promoter (21). Introduction of a galactose-inducible promoter in front of the YCK3 ORF in BJ3505 was as described in ref. 16. pRS426 plasmids containing the N-terminal domain of Vac8 or the Src kinase fused to GFP [Vac8-(1–18)-GFP and Src-(1–16)-GFP] under the control of a NOP1 promoter are from unpublished work (K.S., L.E.P.D., H.H., T.J.L., and C.U.) or were generated by introducing a sequence coding for the first 18 aa of the *Leishmania* protein HASPB [hydrophilic acylated cell surface protein B (22)] with a T6S mutation to allow for myristoylation of the protein into the same vector (our unpublished observations).

Microscopy. Fluorescence microscopy and FM4–64 staining of yeast cells was done as described in ref. 16 and 23. Briefly, yeast cells were grown to $OD_{600} < 0.5$, then incubated with 50 μ M FM4–64 for 1 h, washed twice with medium, and chased for 3 h before being analyzed by fluorescence microscopy. Filter-sterilized synthetic medium/dextrose/complete medium was used for GFP fluorescence of cells grown to mid-log phase. Images were acquired with a Zeiss Axiovert 35 microscope equipped with an AxioCam [filter set 10 (for GFP) or 23 (for FM4–64), $\times 100$ objective] and were processed by using PHOTOSHOP 7.0.

Vacuole Purification and Fusion Assay. Vacuoles were purified and analyzed for fusion as reported in ref. 24. Vacuoles from each tester strain (3 μ g each, 30- μ l total volume) were incubated for the indicated time at 26°C in reaction buffer (125 mM KCl/5 mM $MgCl_2$ /20 mM Pipes/KOH, pH 6.8/200 mM sorbitol), a protease inhibitor mixture (PIC) (7.5 μ M pefabloc SC/7.5 ng/ml leupeptin/3.75 μ M *o*-phenanthroline/37.5 ng/ml pepstatin), and an ATP-regenerating system. One unit of fusion activity equals 1 μ mol of *p*-nitrophenol phosphate hydrolyzed per min per μ g *pep4 Δ* vacuoles at 30°C.

Biochemical Fractionation of Yeast Cells and Vacuoles. Subcellular fractionation and analyses of membrane association were done as described in refs. 16 and 25 (K.S., L.E.P.D., H.H., T.J.L., and C.U., unpublished work). Proteins were analyzed by SDS/PAGE and Western blotting.

Biotinylation Assay. To detect endogenously palmitoylated proteins, a recently developed protocol was applied (7, 26). Cells (20 OD_{600} units) from a logarithmically grown culture were pelleted and resuspended in 300 μ l of lysis buffer [PBS/protease inhibitor mixture (Roche)/5 mM EDTA]. Cells were broken by the addition of 100 μ l of 0.5-mm glass beads with a cell disrupter (4 min at 4°C). Samples were centrifuged (300 $\times g$ for 5 min), supernatants were removed, and the pellet was reextracted. One milligram of pooled supernatants was then incubated with lysis buffer (600 μ l) containing 1% Triton X-100 and 25 mM *N*-ethylmaleimide to quench free cysteines (30 min at 4°C), and proteins were precipitated with methanol/chloroform. The air-dried pellet was resuspended in 100 μ l of resuspension buffer (2% SDS/8 M urea/100 mM NaCl/50 mM Tris-HCl, pH 7.4) by sonication, diluted with 600 μ l of 1 M hydroxylamine (pH 7.4) and 300 μ M biotin-BMCC (Pierce), and rotated for 2 h at 4°C. As a control, hydroxylamine was replaced by PBS. Proteins were then precipitated with methanol/chloroform, dried, and resuspended by sonication as before. Lysis buffer with 0.1% Triton X-100 (1 ml) was added to each sample, aliquots (60 μ l) were removed as loading control, and the remaining reactions were incubated with 100 μ l of neutravidin-agarose beads (Pierce) for

1 h at room temperature on a nutator. Neutravidin beads were washed with PBS containing 0.5 M NaCl and 0.1% Triton X-100 and then once with PBS. Proteins were eluted by boiling for 5 min in 20 μ l of resuspension buffer containing 4 \times SDS sample buffer lacking 2-mercaptoethanol. Samples were analyzed by SDS/PAGE and Western blotting.

Palmitoylation of Proteins on Isolated Vacuoles. Vacuoles from the respective strains (60 μ g) were incubated in fusion reaction buffer without ATP and with [3H]Pal-CoA (27). After 10 min, vacuoles were collected (12,000 $\times g$ for 10 min), resuspended in SDS sample buffer without reducing agents, and analyzed by SDS/PAGE and fluorography.

Results

Identification of a Vacuolar DHHC Protein with a Role in Vac8 Localization. To assess the possible contribution of DHHC proteins to the localization of palmitoylated vacuolar proteins, we analyzed the subcellular localization of Vac8, Meh1/Ego3, and Yck3 in all DHHC deletion strains by subcellular fractionation or fluorescence microscopy. In wild-type cells, all three proteins are found in the vacuole-enriched membrane fraction (Fig. 1A, P, Vac8 and Meh1) or are observed on vacuoles (GFP-Yck3; Fig. 1A). Presence in the supernatant fraction (S) is then indicative of a defective localization of the respective protein. Whereas neither Yck3 nor Meh1/Ego3 are affected by any single DHHC deletion mutant, Vac8 was mislocalized to the supernatant fraction in the *ynl326c/pfa3 Δ* mutant (Fig. 1A). The strength of this defect depends on the strain background: In the EUROSCARF background (BY4741; Fig. 1A), we observed a stronger defect than when we created the *ynl326c/pfa3 Δ* deletion in the vacuole protease-deficient BJ3505 tester strain, where $\approx 50\%$ of Vac8 was membrane-localized (Fig. 1B). A similar Vac8 localization like in the BJ strain was also observed in the second tester strain, DKY6281 (data not shown). To be able to directly assess function in vacuole fusion, to avoid proteolysis, and to allow comparison with several of our Vac8 mutants, we selected the BJ background for further studies and, if possible, compared it with the BY background. We confirmed that Vac8 in the nonvacuolar fraction was indeed soluble in *ynl326c/pfa3 Δ* cells, because it was recovered in the supernatant after high-speed centrifugation (Fig. 1B). Interestingly, we observed a slight size shift between membrane-associated and soluble Vac8. To explore whether this mobility shift reflected differences in the palmitoylation state of the membrane-associated and soluble forms, we analyzed Vac8's mobility on lower percentage gels, comparing *ynl326c Δ* and wild type. We found that each contained both Vac8 forms, with the upper band being exclusively in the membrane fraction. Doublets were lost if samples were boiled in gel loading buffer containing a reducing agent (Fig. 1C), suggesting that Vac8 is still palmitoylated on membranes in *ynl326c Δ* cells. Yck3 localization was similar in both strains (Fig. 1A–C and E). While this study was under way, Linder and colleagues (28) reported a function of Ynl326c in Vac8 palmitoylation. Because they termed the protein Pfa3 (protein fatty acyltransferase 3), we will refer to this name hereafter.

Effect of *pfa3* Deletion on Vacuole Morphology and Inheritance. Pfa3 is localized to the vacuole (<http://yeastgfp.ucsf.edu>) and is therefore an ideal candidate to be involved in palmitoylation of Vac8. We analyzed GFP-tagged Vac8 in BJ wild-type and *pfa3 Δ* cells by fluorescence microscopy. In agreement with our previous observations (Fig. 1), Vac8-GFP was exclusively found on vacuoles in wild-type cells but, consistent with our fractionation data, showed a significant cytosolic staining in *pfa3 Δ* cells (Fig. 1D). This partial mislocalization is in contrast to the exclusively cytosolic presence of a Vac8 mutant, in which all three SH4-domain cysteines have been mutated (29) (K.S., L.E.P.D., H.H.,

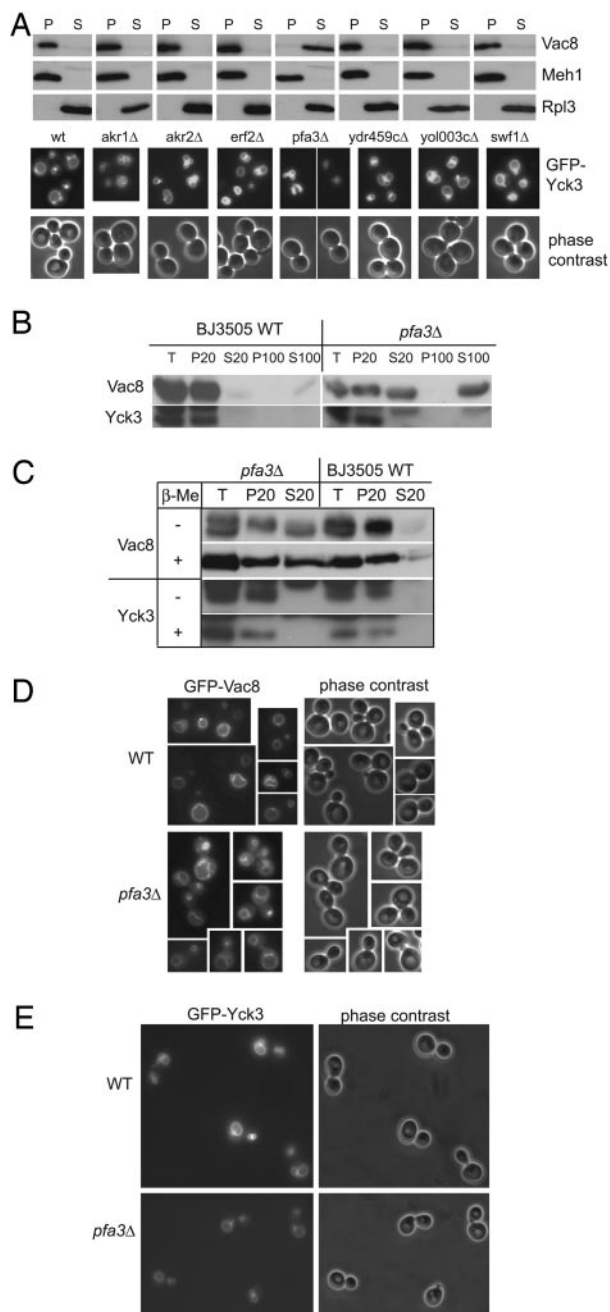


Fig. 1. Analysis of the DHHC mutants for Vac8 and Yck3 localization. (*A Upper*) Subcellular fractionation. Cells from the respective deletion strains (BY4741, EUROSCARF) were lysed and separated by centrifugation ($13,000 \times g$ for 10 min at 4°C) into a pellet (P) and supernatant fraction (S) as described in *Methods*. Proteins were analyzed by SDS/PAGE and Western blotting with antibodies to Meh1, Vac8, and Rpl3, a ribosomal subunit. (*A Lower*) The indicated strains expressing GFP-Yck3 were analyzed by fluorescence microscopy. (*B*) Subcellular fractionation in BJ3505 cells. The lysate was centrifuged for 10 min at $20,000 \times g$ to yield the P20 and S20 fractions. To get the P100 and S100 fractions, a similar amount of the S20 fraction was spun again ($100,000 \times g$ for 30 min at 4°C). Proteins in pellet and TCA-precipitated supernatant were analyzed by SDS/PAGE and Western blotting with antibodies to Vac8 and Yck3. The top band in the Yck3 illustration corresponds to Vac8, which was detected before Yck3. (*D* and *E*) Localization of GFP-tagged Vac8 and Yck3 in BJ wild-type and *pfa3Δ* cells. Yeast strains expressing the indicated GFP variant were analyzed by fluorescence microscopy.

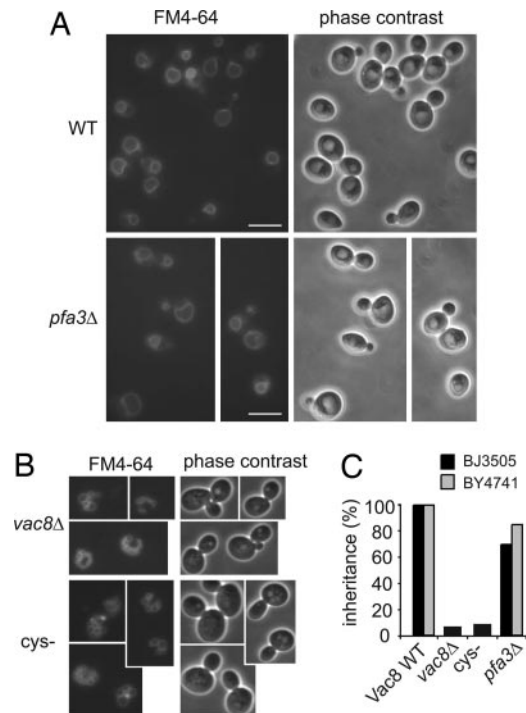


Fig. 2. Vacuole morphology and inheritance. (*A* and *B*) Vacuoles from the indicated BJ wild-type, *pfa3Δ*, *vac8Δ*, and the Cys- mutant, which expresses a Vac8 mutant lacking the N-terminal cysteines (Cys- is Vac8-C_{4,5,7A}) were stained with $50 \mu\text{M}$ FM4-64 and observed by fluorescence microscopy. (*C*) Vacuole inheritance was scored for the indicated strains as described in *Methods*.

T.J.L., and C.U., unpublished work). Furthermore, vacuole morphology was not affected in *pfa3Δ* cells (Fig. 2*A*). However, we note that again, consistent with the strain-dependent differences in Vac8 fractionation, *pfa3Δ* vacuoles in BY were partially fragmented, as were *akr1Δ* and *akr2Δ* cells (Fig. 1*A*). It is presently unclear whether either *akr1* or *akr2* deletion affects palmitoylation of vacuolar proteins. It is noteworthy that BY strains have in general more vacuoles than our tester strains. Loss of Vac8 or a mutation of all target cysteines in the SH4 domain (Cys-) leads to complete vacuole fragmentation (Fig. 2*B*). Moreover, when we scored *pfa3Δ* for vacuole inheritance, we observed only a 10–20% defect in either BJ or BY cells, whereas cells lacking Vac8 or the Vac8 Cys mutant are completely deficient in vacuole inheritance (Fig. 2*C*) (K.S., L.E.P.D., H.H., T.J.L., and C.U., unpublished work). These data suggest that Pfa3 plays an important, but not exclusive, role in Vac8's localization to the vacuole. Because vacuole inheritance requires Vac8 palmitoylation (K.S., L.E.P.D., H.H., T.J.L., and C.U., unpublished work), it is possible that other proteins, such as Ykt6, compensate sufficiently for the lack of Pfa3.

Pfa3 Affects Vacuole Fusion and Vac8 Palmitoylation. Vac8 requires palmitoylation for localization to vacuoles, vacuole inheritance, and efficient participation in the fusion reaction. We therefore analyzed the role of Pfa3 in *in vitro* vacuole fusion. We deleted *PFA3* from both tester strains and used them in the fusion reaction (30). Vacuoles lacking Pfa3 showed a 50% reduction in fusion (Fig. 3*A*), consistent with a defective Vac8 localization (Fig. 1). However, fusion is still functional if Vac8 is partially localized (K.S., L.E.P.D., H.H., T.J.L., and C.U., unpublished work), indicating that Pfa3 is likely to affect additional proteins beside Vac8. In addition, fusion was still sensitive to all inhibitors of the fusion reaction, including Ykt6 and Vac8 antibodies (Fig.

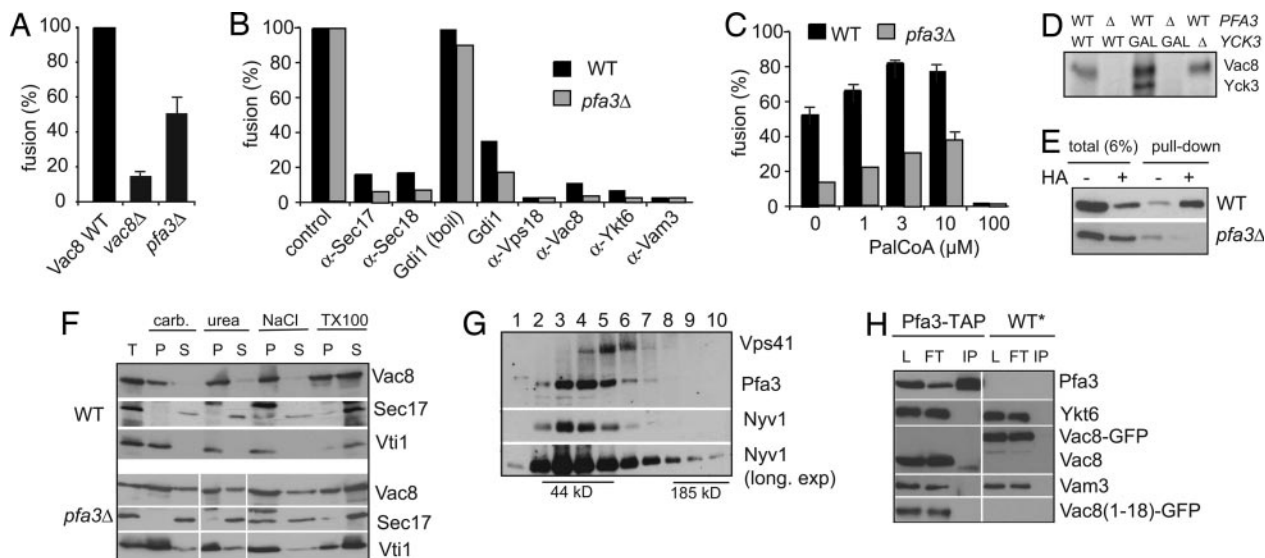


Fig. 3. Functional analysis of *pfa3* deletion. (A) Vacuole fusion. Vacuoles from tester strains with the respective deletion were incubated at 26°C for 90 min and then analyzed for fusion (SD, $n = 3$). Fusion deficiency is stronger in the DKY background than in BJ (not shown). For details see *Methods*. (B) Sensitivity to inhibitors. Fusion was analyzed as in A in the presence or absence of the indicated antibodies. All antibodies were titrated into the reaction (not shown), and the strongest inhibition is shown. In general, antibody concentration was at $\approx 100 \mu\text{g/ml}$. For Gdi1, the inhibitor was boiled to determine the buffer control. The control reaction was set to 100% for each combination. For additional information, see ref. 16. (C) Effect of Pal-CoA on fusion. Increasing Pal-CoA amounts were titrated into the fusion reaction, and fusion was determined as before. (D) Palmitoylation of Vac8 and Yck3 on vacuoles. Purified vacuoles ($60 \mu\text{g}$) from the respective strain were incubated in fusion reaction buffer with [^3H]Pal-CoA for 10 min at 26°C. Vacuoles were then collected ($12,000 \times g$ for 10 min at 4°C), and proteins were analyzed by SDS/PAGE and fluorography. (E) Palmitoylation determined by the biotin-switch method. The indicated cells were lysed, free cysteines were quenched by *N*-ethylmaleimide, and the extract was subjected to the biotin-switch procedure by using hydroxylamine (HA) and biotin-BMCC as a crosslinker. Crosslinked proteins were captured on Neutravidin agarose (Pierce), eluted by boiling, and analyzed by SDS/PAGE. Western blots were decorated with antibodies to Vac8. Total (6%) refers to the fraction of lysate removed before the Neutravidin pull-down. (F) Membrane association of Vac8. Vacuoles from the indicated strains ($30 \mu\text{g}$ each) were diluted into $500 \mu\text{l}$ of 20 mM Hepes/KOH (pH 7.4), 1 mM PMSF, and the following conditions: 0.1 M Na_2CO_3 (carb.), 6 M urea, 1 M NaCl, or 1% Triton X-100, and incubated for 30 min on ice. Samples were then centrifuged ($100,000 \times g$ for 30 min at 4°C) and split into pellet (P) and supernatant (S). An aliquot corresponding to the membrane fraction used in each condition is shown for comparison (T). Proteins in pellet and TCA-precipitated supernatant were analyzed by SDS/PAGE and Western blotting. (G) Sizing of Pfa3. Vacuoles ($200 \mu\text{g}$) from BJ3505 carrying Pfa3-TAP were incubated for 10 min at 4°C in lysis buffer (20 mM Tris-HCl, pH 7.4/150 mM NaCl/0.5% Triton X-100/1 \times PIC), insoluble material was removed by centrifugation ($20,000 \times g$ for 10 min at 4°C), and the detergent extract was loaded onto a linear 10–30% glycerol gradient prepared in lysis buffer. The sample was then centrifuged ($40,000 \text{ rpm}$ in an SW40 rotor for 18 h at 4°C), 0.5-ml samples were collected from the top of the gradient, and TCA-precipitated proteins were analyzed by SDS/PAGE and Western blotting. The first 10 samples are shown. Vps41 (120 kDa) is the only protein that had a second peak at the bottom of the gradient (not shown); the SNARE Nyv1 (30 kDa) is found in a complex at 105 as part of the SNARE complex (26). (H) Physical associations. The detergent lysate from a BJ Pfa3-TAP strain expressing Vac8(1–18)-GFP was prepared as in G and incubated with IgG beads overnight at 4°C. Load (L) and flow-through (FT) (1% each) were removed, and proteins were TCA-precipitated. Beads were washed two times in lysis buffer. Proteins were then eluted by boiling in sample buffer and analyzed as before. A BJ *vac8* Δ strain expressing Vac8-GFP (WT*) served as a negative control. The lower band in the Vac8 illustration is due to the Pfa3-TAP signal, which runs at a similar molecular weight.

3B), and was stimulated in either case by palmitoyl-CoA (Fig. 3C). Previously, we reported that Vac8 palmitoylation occurs in an early subreaction on vacuoles that is sensitive to antibodies to Sec18 and Ykt6 (14, 19). We therefore incubated vacuoles with [^3H]Pal-CoA and analyzed Vac8 palmitoylation. Only on wild-type vacuoles did we observe efficient palmitoylation (Fig. 3D); *pfa3* Δ vacuoles did not show labeling of Vac8. To find out whether the lack of palmitoylation on *pfa3* Δ vacuoles is Vac8-specific, we analyzed cells in which Yck3 is overexpressed, which is required to detect its *in vitro* palmitoylation (16). Although palmitoylation of both Vac8 and Yck3 was clearly detectable in the Yck3 overexpression background, neither protein was labeled in the absence of Pfa3. Because Yck3 is localized to vacuoles in *pfa3* Δ cells (Fig. 1), it is possible that overexpression allows us to monitor a vacuolar palmitoylation event that does not normally occur. In wild-type or *pfa3* Δ cells, most Yck3 might already be palmitoylated before reaching the vacuole; the current model is that this also requires the DHHC protein Akrl at the Golgi (15). When Yck3 is overexpressed, some of it might be recruited directly from the cytoplasm to the vacuole, where it would then become a substrate for Pfa3-dependent palmitoylation. Consistent with this possibility, we found that truncated

forms of Yck3 that lack its C-terminal cysteines only become vacuole-localized when overexpressed (unpublished observations). Intriguingly, we also found that Yck3 is also sorted to vacuoles when *akr1* has been deleted (Fig. 1A), suggesting that Akrl and Pfa3 play compensatory roles in Yck3 palmitoylation. Vac8's palmitoylation might similarly be affected by multiple proteins, i.e., Pfa3 and Ykt6. To determine whether the lack of *in vitro* Vac8 palmitoylation in *pfa3* Δ is due to its inaccessibility at the vacuole, we conducted an experiment to detect its endogenous palmitoylation state using the biotin-switch method. For this, proteins were first incubated with *N*-ethylmaleimide to quench free cysteines, and then treated with hydroxylamine to cleave the thioester. The free cysteine was then crosslinked to biotin, and proteins were collected by neutravidin pull-down. Our data indicate that Vac8 palmitoylation is detectable in wild-type but severely reduced in *pfa3* Δ cells (Fig. 3E). We therefore conclude that Pfa3 is important for Vac8 palmitoylation on vacuoles, which could then explain the fusion defect. These results are not, however, consistent with the presence of a significant portion of Vac8 on *pfa3* Δ vacuoles. Is Vac8 attached to vacuoles through protein-protein interactions rather than palmitoylation? Or is it possible that the Biotin-switch method

fails to detect certain palmitates if the thioester is rapidly reversed during cell lysis, and some Vac8 is indeed palmitoylated on *pfa3Δ* vacuoles? We therefore analyzed Vac8's hydrophobic properties by subjecting wild-type and *pfa3Δ* vacuoles to extraction with carbonate, urea, or salt, and found that, in all cases, at least 50% of Vac8 remained membrane-associated (Fig. 3F). Only with Triton X-100 was most Vac8 released into the soluble fraction, a result that is typical for integral membrane proteins (like the SNARE Vti1). As a control, the peripheral membrane protein Sec17 was extracted to the supernatant fraction in either case. Thus, Vac8 behaves like a hydrophobic (Fig. 3) and palmitoylated (Fig. 1) protein in *pfa3Δ* cells.

We then analyzed the biochemical properties of the Pfa3 protein. Sizing of epitope-tagged Pfa3 indicates that the majority of the protein is not part of a large complex (Fig. 3G); its migration corresponds to its molecular weight. In agreement with this, Pfa3 does not copurify other vacuolar proteins like Ykt6, Vac8, or Vam3 under the selected conditions (Fig. 3H). Thus, modification of Vac8 or regulation of Pfa3 via other proteins may be mediated by a transient interaction of the proteins.

Role of Pfa3 in Palmitoylation of SH4 Domains. Because Pfa3 is involved in Vac8's vacuolar localization and palmitoylation, we asked whether its function could be assigned to recognition of the SH4 domain, an N-terminal myristoylation motif linked to palmitoylated cysteines or a polybasic stretch. We have shown in a parallel study that the first 16–18 aa of both Vac8 and Src kinase are sufficient to direct GFP to the plasma membrane and to internal membranes (K.S., L.E.P.D., H.H., T.J.L., and C.U., unpublished work). In contrast to Vac8, the N-terminal Src sequence is not palmitoylated but rather anchored by its myristate/polybasic stretch. We used this system to determine whether Pfa3 is required for their membrane localization. The two fusion proteins localize similarly in wild-type or the *swf1Δ* cells (among the other DHHC deletions). In the *pfa3Δ* mutant, however, both were found exclusively at the plasma membrane (Fig. 4A and B). The fact that both types of SH4 domains require Pfa3 for their vacuole localization [Src(1–16)-GFP is not palmitoylated] indicates that Pfa3 also functions as a vacuole-sorting protein. Importantly, one reporter protein containing the SH4 domain from the *Leishmania* highly acidic surface protein B (HASP B, with a T6S mutation to allow for myristoylation) is localized to intracellular membranes and the plasma membrane even in the *pfa3Δ* mutant (Fig. 4C). Regardless of the localization, all cysteine-containing SH4 fusion proteins were palmitoylated *in vivo* (Fig. 4D). Thus, Pfa3 confers localization of fusion proteins with minimal sorting motifs to vacuole membranes. Potentially, Pfa3 recognizes specific amino acids or structural motifs in the Vac8 and Src sequence, which are absent in HASPB (Fig. 4E).

Discussion

In this article, we describe the function of the vacuole-localized DHHC protein, Pfa3. Pfa3 is required for efficient Vac8 localization to vacuoles, consistent with its ability to promote palmitoylation *in vitro* upon reconstitution (28). Loss of Pfa3 leads to partial mislocalization of Vac8 to the cytosol and a reduction in vacuole fusion. In *pfa3Δ* cells, the population of Vac8 that is still localized to vacuoles is apparently palmitoylated, indicating that other palmitoylation factors can substitute for the loss of Pfa3 function. Our studies on Vac8 and Yck3 demonstrate that palmitoylation is affected by a complex network of factors involved in protein sorting and, perhaps redundantly, palmitoyl transfer.

Despite this complexity, the identification of Pfa3 adds to our picture on palmitoylation at the vacuole. Previous studies identified the SNARE Ykt6 as a factor that promotes Vac8 palmitoylation *in vitro* (19). Antibodies to Ykt6 or the N-terminal

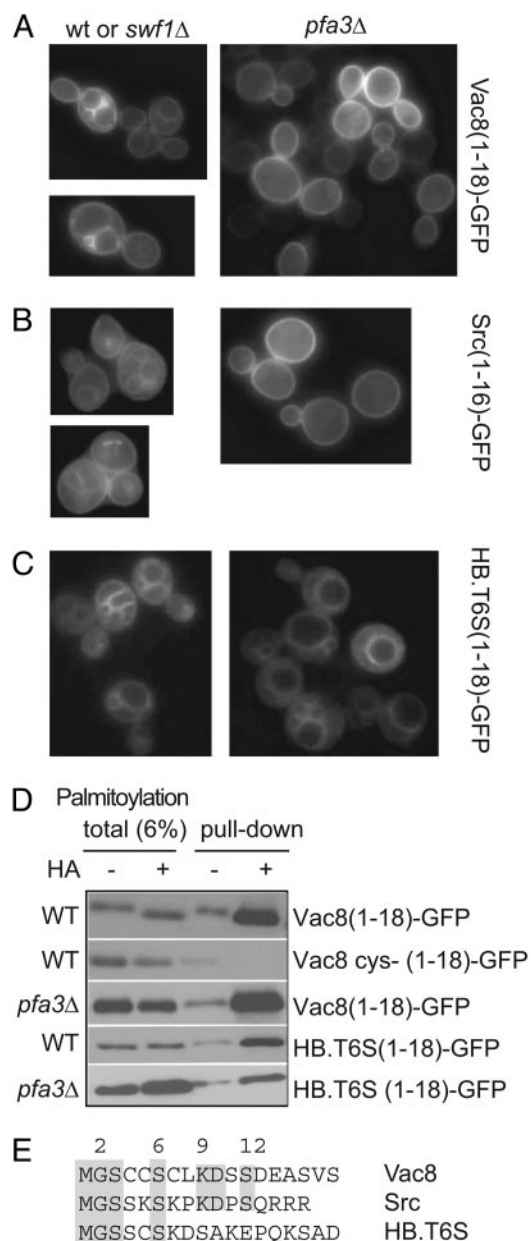


Fig. 4. Localization of minimal targeting constructs in yeast. Vac8(1–18)-GFP (A), Src(1–16)-GFP (B), or HASPB.T6S(1–18)-GFP (HB.T6S) (C) were expressed in BY wild-type or all DHHC deletion strains and analyzed by fluorescence microscopy. Localization was similar in wild type and most DHHC deletion strains (A–C Left). Only in *pfa3Δ* cells exclusive plasma membrane staining for Vac8(1–18) and Src(1–16) was visible (A and B Right). (D) Palmitoylation of the SH4(1–18)-GFP constructs. Palmitoylation of the indicated constructs was determined as described in Fig. 3E. Total (6%) corresponds to the fraction of lysate removed before the Neutravidin pull-down. Blots were decorated with antibodies to GFP. (E) Alignment of the N-terminal 15 aa of the Vac8, Src, and HASPB-T6S sequence.

longin domain interfered with palmitoylation at an early stage of fusion. Because *in vitro* palmitoylation required equimolar amounts of Ykt6 to modify Vac8, we suggested that acylation might occur by a nonenzymatic transfer mechanism (3, 19). Such a mechanism has been suggested before and would explain previous autoacylation data (31). Initial data on the biochemical properties of DHHC-CRD protein pointed to the key function of the DHHC motif in palmitoylation but also showed similar acyl transfer properties as observed for Ykt6 (8, 10). However,

more recent studies on DHHC-dependent palmitoylation have demonstrated enzymatic activity with turnover numbers ranging between three per min for Ras (13) and two per h for Vac8 (31). This rate is still very poor for Vac8, suggesting that additional factors like Ykt6 and Sec18 increase efficiency of this reaction *in vitro*. We previously showed that the palmitoylation activity for Vac8 is present in a low-molecular-weight fraction prepared from detergent-solubilized vacuoles (19). Because both Ykt6 and Pfa3 migrate in this fraction, it is possible that both activities in the detergent extract contributed to Vac8 palmitoylation *in vitro* (32). Taken together, our data suggest that palmitoylation of both Vac8 and Yck3 is not under the control of a single protein but rather involves coordination among factors that regulate their localization and accessibility to different palmitoylation factors en route to the vacuole.

A protein's palmitoylation does not necessarily need to occur at its destination compartment. Swf1 appears to modify the endosomal SNARE Tlg1 at the ER, the Golgi protein Akr1 acts on the plasma membrane kinase Yck2, and the ER-localized Erf2 palmitoylates Ras2, which is found at the Golgi and the plasma membrane (7, 9, 10). The presence of palmitoyl transfer proteins/palmitoyltransferases at multiple organelles raises the important issue of how the specificity of palmitoylation reactions is conferred. Potentially, DHHC proteins recognize not only a sequence motif but also a substrate's structure and/or residence in a certain complex, which would serve as a palmitoylation signal. That the substrate provides the signal makes sense, because palmitoyl transfer proteins/acyltransferases seem to have overlapping activities that can compensate for one another. According to our observations, none of the single DHHC deletions resulted in mislocalization of Yck3 or Meh1, but both proteins depend on palmitoylation for vacuole binding (ref. 15 and unpublished observations). Also, deletion of *ERF2* still

permits partial Ras2 palmitoylation and localization (33), and our data suggest that some Vac8 is palmitoylated in *pfa3Δ* cells. In agreement with this postulate, Linder and colleagues (28) detected residual palmitoylation of Vac8 by metabolic labeling with [³H]palmitate in *pfa3Δ* cells. The localization of Vac8 to *pfa3Δ* vacuoles is in striking contrast to the poor binding of a Vac8 mutant that lacks its N-terminal cysteines and thus cannot be palmitoylated (K.S., L.E.P.D., H.H., T.J.L., and C.U., unpublished work). We postulate that other proteins (DHHC family members or Ykt6) support Vac8's palmitoylation in *pfa3Δ* cells. In this mutant, Vac8 may not be palmitoylated at all three possible cysteines or certain cysteines may also be more prone to deacylation. Deacylation of certain cysteines, which would be quenched during the *N*-ethylmaleimide treatment during the biotin-switch procedure, might explain why we failed to detect labeling of Vac8 in *pfa3Δ* cells using this method.

In summary, we presented evidence that Pfa3 is a factor that affects palmitoylation of Vac8 on yeast vacuoles. Our data suggest that Pfa3 targets proteins to the vacuole by recognizing certain amino acids or structural motifs within the SH4 motif, which then facilitates their efficient palmitoylation. Further work will be needed to determine the specificity, regulation, and redundancy of factors that function in the palmitoylation of vacuolar proteins.

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