Supplementary materials

Yeast strains and molecular biology

Yeast strains

To generate Ykt6 mutants a diploid yeast strain carrying a Ykt6 deletion on one allele (BY4743; *MAT a/alpha; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 YKL196c::kanMX4/YKL196c*; EUROSCARF) was transformed with a centromeric pRS416 (URA3) vector encoding Ykt6 under its original promotor. Yeast cells were then sporulated for three days on SPOR plates (0.02% (w/v) raffinose, 0.3% (w/v) KAc) and tetrads were dissected. The resulting haploid cells carrying a *ykt6* deletion complemented by the *pRS416-YKT6pr-YKT6* plasmid (CUY434) were then transformed with pRS413 (HIS3, CEN) or pRS423 (HIS3, 2μ) vector encoding the respective mutants of Ykt6 and selected on SDC-his/-ura. If the mutants were sufficient for survival the *pRS416-YKT6pr-YKT6* vector was removed by growing the cells on 5-fluoroorotic acid (5-FOA). Otherwise both plasmid were maintained by growing the cells in SDC–his/-ura. The Nyv1 transmembrane anchor was replaced by the CCIIM anchor and TAA codon after amino acid 232 by genomic tagging; primers were used to amplify a kanMX-marker from pFA6-kanMX6 (Longtine et al., 1998) that inserted by homologous recombination at the region coding for the Nyv1 transmembrane domain. *APT1* was deleted by transforming wild-type strains with a PCR-amplified URA3 marker with flanking *APT1* sequences to generate BJ3505 a*pt1*Δ and DKY6281 *apt1*Δ (Longtine et al., 1998). All other strains were as described in Dietrich et al., 2004.

Plasmids and recombinant proteins

To generate *YKT6*pr the 800 nucleotides upstream of the *YKT6*start codon were PCR-amplified with proofreading polymerase (Eppendorf) using primers with EcoR1 (5') and BamH1 (3') restriction sites. The fragment was ligated into EcoR1/BamH1 digested pRS413 and pRS416.

To make *YKT6*, *Ykt6*^{ACIIM}, *Ykt6*^{CAIIM}, and *Ykt6*^{AAIIM} the *YKT6* ORF was amplified using primers with BamH1 (5') and Sac1 (3') sites. The 3' primers encoded for wild-type or modified C-terminal amino acids (CCIIM, ACIIM, CAIIM, AAIIM). All fragments were BamH1/Sac1 inserted into *pRS413-YKT6pr*. In addition, the YKT6 fragment was BamH1/Sac1 inserted into *pRS416-YKT6pr*.

To generate *pRS423-YKT6pr-Ykt6*^{GFP} first an internal BamH1 restriction site was created in YKT6. For this two fragments were amplified: The first contains 800 nucleotides upstream of YKT6's start codon and the first 357 nucleotides of YKT6 (flanked by EcoR1and BamH1 sites). The second fragment contains nucleotides 361 to 603 of YKT6 (flanked by BamH1and Sac1 sites). The fragments were ligated into EcoR1/Sac1 digested *pRS423*. The vector was then BamHI digested, and one copy of a BamH1/BglII eGFP cassette, digested from pBS-eGFP (Ed Hurt, Heidelberg, Germany), was inserted. The correct orientation of the inserts was verified by restriction digest.

GFP$_{int}$*YKT6*, *GFP*$_{int}$*Ykt6*^{ACIIM}, *GFP*$_{int}$*Ykt6*^{CAIIM}, and *GFP*$_{int}$*Ykt6*^{AAIIM} were then generated by PCR-amplification of the *YKT6* ORF using primers with BamH1 (5') and EcoR1 (3') sites and *pRS413-YKT6pr-GFP*$_{int}$*Ykt6* as template. The 3' primers encoded for wild-type or modified C-terminal amino acids (CCIIM, ACIIM, CAIIM, AAIIM). All fragments were BamH1/Sac1 inserted into *pRS423-YKT6pr*.

For overexpression in yeast, YKT6 and Ykt6^{AAIIM} were PCR amplified from the YKT6 ORF using a 5' primer with Sph1 and an *His*-tag and 3' primers (BamH1) encoding for wild-type or modified C-terminus (AAIIM). Fragments were ligated into pGALPatG1L.

For overexpression in *E. coli*, Ykt6 mutant genes were PCR amplified from the above-mentioned vectors using primers containing BamH1 (5') or HindIII (3') restriction sites. The fragments were inserted into a BamH1/HindIII digested pQE30 vector (*Ykt6*^{AAIIM}). Proteins were overexpressed in *E.coli* (BL21) in the presence of 1 mM IPTG and purified by Ni-NTA affinity chromatography or gluathione-coated beads as previously described (Veit et al., 2001). Neither tag affected Vac8 or self-palmitoylation activity of Ykt6 *in vitro* (not shown).

All other plasmids and recombinant proteins were as described in Dietrich et al., 2004.

Figures and Tables

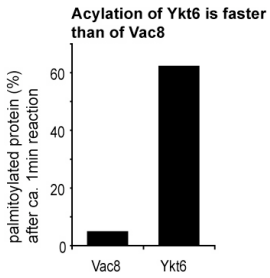

Figure S1. Ykt6 palmitoylation precedes Vac8 palmitoylation. The palmitoylation reaction as in Figure 1A was stopped after 1 min, and samples were processed as described. The signals were quantified by using NIH Image 1.63. The signals at 1 min are shown as a percentage of the signals at 30 min (100%).

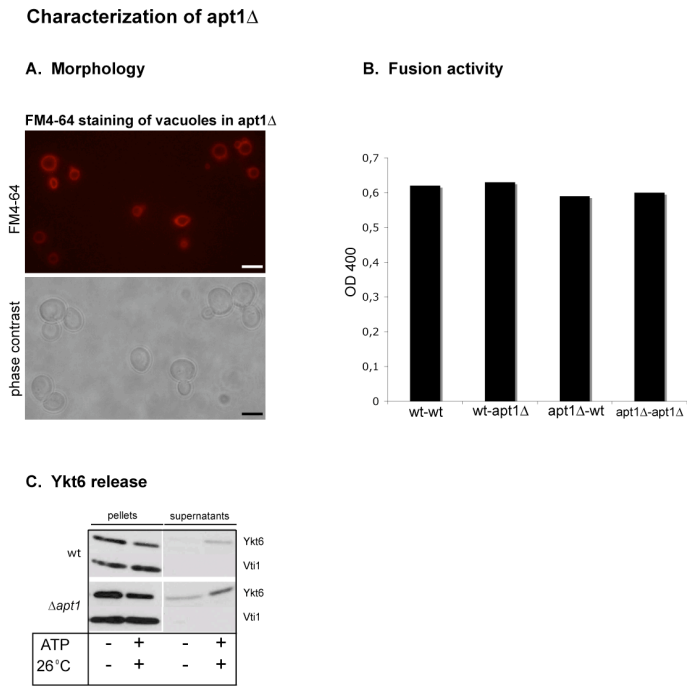

Figure S2. Characterization of *apt1*Δ. (A) *In vivo* labeling of *apt1*Δ vacuoles by pulse-chase with FM4-64 (Vida and Emr, 1995). (B) Isolated vacuoles deleted for *apt1* were fused *in vitro*, where OD_{400} reflects the fusion activity (Haas, 1995); deletion strains were fused against wild-type reporter strains in the combinations indicated. (C) Protease-deficient (BJ3505) wild-type or *apt1*Δ vacuoles were assayed for Ykt6 release, as in Figure 4.

Table S1

S. cerevisiae strains used in this study

Strain	Genotype	Reference
BJ3505	*MATa pep4Δ::HIS3 prb1-Δ1.6R HIS3 lys 2-208 trp1-D101 ura3-52 gal2 can*	Haas 1995
DKY6281	*MATa leu2-3 leu2-112 ura3-52 his3-delta200 trp 1-D101 lys 2-801 suc 2-D9 pho8 :: TRP1*	Haas 1995
BY4743	*MAT a/alpha; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0 YKL196c::kanMX4/YKL196c*	EUROSCARF
CUY322	BJ3505; *APT1::URA3*	this study
CUY324	DKY6281; *APT1::URA3*	this study
CUY434	*MATa; his3Δ1; leu2Δ0 ura3Δ0 YKL196c::kanMX4/YKL196c*; *pRS416-YKT6pr-YKT6*	this study
CUY579	CUY434; *pRS413- YKT6pr-YKT6*	this study
CUY581	CUY434; *pRS413-YKT6pr-Ykt6* ^{CAIIM}	this study
CUY580	CUY434; *pRS413- YKT6pr-Ykt6* ^{ACIIM}	this study
CUY587	CUY434; *pRS413- YKT6pr-Ykt6* ^{AAIIM}	this study
CUY437	BY4743 *pep4Δ::HIS3*; *pGALPatG1L-His-YKT6*	this study
CUY569	BY4743 *pep4Δ::HIS3*; *pGALPatG1L-His-Ykt6* ^{AAIIM}	this study
CUY723	BJ3505*; vam3Δ::TRP1, ura3::pRS406-Vam3pr-VAM3-CCIIM*	Rohde et al., 2003
CUY724	BJ3505*; NYV1::CCIIM-kanMX6*	this study
CUY996	CUY434; *pRS423- YKT6pr- GFP*$_{int}$ *Ykt6* ^{CAIIM}	this study
CUY997	CUY434; *pRS423-YKT6pr- GFP*$_{int}$ *Ykt6* ^{ACIIM}	this study
CUY998	CUY434; *pRS423- YKT6pr- GFP*$_{int}$ *Ykt6* ^{AAIIM}	this study
CUY1208	CUY434; *pRS423- YKT6pr-GFP*$_{int}$*YKT6*	this study

References:

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Veit, M., Laage, R., Dietrich, L., Wang, L. and Ungermann, C. (2001) Vac8p release from the SNARE complex and its palmitoylation are coupled and essential for vacuole fusion. *Embo J*, 20, 3145-3155.

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