Vac8p, an Armadillo Repeat Protein, Coordinates Vacuole Inheritance With Multiple Vacuolar Processes

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Vac8p, an armadillo (ARM) repeat protein, is required for multiple vacuolar processes. It functions in vacuole inheritance, cytoplasm-to-vacuole protein targeting pathway, formation of the nucleus–vacuole junction and vacuole–vacuole fusion. These functions each utilize a distinct Vac8p-binding partner. Here, we report an additional Vac8p function: caffeine resistance. We show that Vac8p function in caffeine resistance is mediated via a newly identified Vac8p-binding partner, Tco89p. The interaction between Vac8p and each binding partner requires an overlapping subset of Vac8p ARM repeats. Moreover, these partners can compete with each other for access to Vac8p. Furthermore, Vac8p is enriched in three separate subdomains on the vacuole, each with a unique binding partner dedicated to a different vacuolar function. These findings suggest that a major role of Vac8p is to spatially separate multiple functions thereby enabling vacuole inheritance to occur concurrently with other vacuolar processes.

**Key words:** organelle division, organelle inheritance, Vac8p, Vac17p, yeast

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Vac8p contains 11 armadillo (ARM) repeats (7–9), tandem imperfect repeats of ∼42 amino acids (10). The ARM repeats share a conserved three-dimensional structure. Each ARM repeat forms three α-helices. Tandem ARM repeats fold over each other and form a superhelix. This generates an extended surface for protein–protein interactions (11). Vac8p is both myristoylated and palmitoylated; the acylation is required for targeting the Myo2p–Vac17p–Vac8p complex to the vacuole membrane (12).

The ARM repeat proteins have been identified in plants, fungi and animals (10). One of the best studied ARM proteins β-catenin interacts with more than 20 proteins and plays essential roles in the maintenance of cell–cell adhesions and in the Wnt signaling pathway (13). At the plasma membrane, β-catenin interacts with E-cadherin. In the cytoplasm, in the absence of Wnt signaling, β-catenin is specifically degraded via its interaction with a destruction complex that includes adenomatous polyposis coli (APC). In the presence of Wnt signaling, degradation is blocked, and β-catenin translocates into the nucleus where it interacts with the transcription factor LEF/Tcf (lymphoid enhancer factor/T cell factor) (13). The crystal structures of β-catenin in separate complexes with E-cadherin, APC and LEF-1/Tcf demonstrate that multiple residues on β-catenin are required for its interactions, and that each partner binds to overlapping regions (14–19).

Vac8p is not a homologue of β-catenin, but like β-catenin, it binds to several protein partners, and thereby plays essential roles in multiple cellular processes (3–7,20). Vac8p–Vac17p interaction is required for Myo2p association with the vacuole and subsequent vacuole movement (1,2). Vac8p–Apg13p interaction is required for the Cvt pathway and is specifically required for closure of Cvt vesicles (3). Vac8p–Nvj1p interaction is required for the formation of the nucleus–vacuole junction, a tight apposition of a region of the outer nuclear membrane with the vacuole membrane. The nucleus–vacuole junction is proposed to be a site where a part of the nucleus is engulfed prior to degradation in the vacuole (4,20). Vac8p is also required for homotypic vacuole–vacuole fusion; however, the binding partner(s) for fusion remain to be identified (5,6).

Here, we identify five new putative Vac8p-binding partners. Among them is Tco89p, a binding partner required for caffeine resistance, a new function of Vac8p. It appears that each Vac8p-interacting protein is dedicated to a single Vac8p-related function. Moreover, each binding partner interacts with an overlapping subset of Vac8p ARM repeats.
repeats, suggesting that the binding of these partners is mutually exclusive. This mutual exclusivity combined with the localization of Vac8p to multiple, distinct subdomains on the vacuole membrane may be part of a mechanism whereby Vac8p is required for vacuole movement during the cell cycle and is simultaneously engaged in other vacuole-related functions.

**Results**

**Vac17p is solely required for vacuole inheritance**

Vac8p and Vac17p function together in vacuole movement (1,2,7). Vac8p is also required for several additional processes. Therefore, we tested whether the Vac8p-binding protein, Vac17p, is required for Vac8p functions that are not directly related to vacuole movement. In wild-type cells, Nvj1p-green fluorescent protein (GFP) is confined to a region on the nuclear membrane where the nuclear envelope is in direct contact with the vacuole membrane; this localization requires Vac8p (4) (Figure 1A). However, Vac17p is not required for this localization; in vac17Δ cells, Nvj1p localizes properly to the nucleus–vacuole junction (Figure 1A,B). We also tested whether Vac17p is required for homotypic vacuole–vacuole fusion. In the absence of Vac8p, vacuoles are fragmented. However, the vacuole morphology of vac17Δ is similar to that of wild-type cells (Figure 1A), demonstrating that vacuole–vacuole fusion is normal. Third, we tested whether Vac17p is required for the Cvt pathway, whereby the precursor of vacuolar aminopeptidase (prApe1p) is imported into the vacuole. Upon its arrival in the vacuole, prApe1p is matured to Ape1p (3,7). In vac8Δ cells, the Cvt pathway is blocked and only prApe1p is observed. Deletion of Vac17p had a minor effect on the maturation of Ape1p (Figure 1C).

In addition to the previously identified Vac8p-related functions, we also discovered that vac8Δ mutants are sensitive to caffeine. Thus, we tested if vac17Δ is also caffeine sensitive. The growth of vac8Δ cells was completely inhibited in the presence of 0.2% caffeine, while vac17Δ cells showed a partial sensitivity (Figure 1D). Taken together, these results show that while both Vac17p and Vac8p are required for vacuole inheritance, Vac17p is not critical for other known functions of Vac8p. Likewise, the other previously characterized binding partners of Vac8p, Nvj1p and Apg13p, are not required for vacuole inheritance (3,4).

**Identification of additional Vac8p-binding partners**

A yeast two-hybrid screen for Vac8p-binding partners identified eight open reading frames (ORFs) (Table 1). Three of the eight ORFs Vac17p (2), Apg13p and Vab2p (Vac8p-binding protein 2) (4,23) were identified previously. A ninth ORF, Nvj1p, was not obtained in this screen, but was previously identified as a Vac8p-binding partner (4) and thus was included in our subsequent analyses (Table 2).

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**Figure 1:** While Vac8p is required for multiple vacuolar processes, Vac17p is solely required for vacuole inheritance.

A and B) Nucleus–vacuole junctions in wild-type, vac8Δ and vac17Δ cells. Cells carrying pNVJ1-2XGFP were labeled with FM4-64 for 1.5 h and chased for one doubling time. Red (vacuole) and green (Nvj1p-2XGFP) were merged to show the localization of Nvj1p relative to the vacuole membrane. The formation of nucleus–vacuole junctions (NVJ) was reported as the ratio of the number of cells where Nvj1p localized to a crescent bar or spot between the nucleus and vacuole versus the number of total cells with detectable GFP fluorescence. More than 50 GFP-containing cells were scored for each strain. Scale bar is 5 μm. C) Maturation of aminopeptidase I (Ape1p) in wild-type, vac8Δ and vac17Δ cells. Crude extracts from ~0.2 OD600 of cells were loaded onto 10% SDS-PAGE. Immunoblot analysis using anti-Ape1p antibodies was performed. D) Caffeine resistance of wild-type, vac8Δ and vac17Δ cells. Log-phase cells were spotted onto YEPD (yeast extract-peptone-dextrose) and YEPD+0.2% caffeine plates and incubated at 24°C for 2 and 4 days, respectively.
known function for Vid21p is as a component of the NuA4 wall (26). Despite this overlap in phenotypes, the current glucan, two of the four major components of the yeast cell vid21 and The other new candidate partner is Vid21p. Both of cell wall integrity (21). reported to be a member of the yeast Tor (21,23). Tco89p was recently sensitive to caffeine (Table 2). Moreover, Tco89p is found on the vacuole membrane (21,23). Tco89p was recently reported to be a member of the yeast Tor (target of rapamycin) complex 1 and is required for the maintenance of cell wall integrity (21).

The other new candidate partner is Vid21p. Both vac8l and vid21 have elevated levels of β-1,6-glucan and β-1,3-glucan, two of the four major components of the yeast cell wall (26). Despite this overlap in phenotypes, the current known function for Vid21p is as a component of the NuA4 histone acetyltransferase complex (22). It remains to be determined whether Vid21p has a second localization and function as a Vac8p-binding partner.

These potential Vac8p-binding partners that had not been previously analyzed were deleted from LWY7235 (wild type) and the corresponding knockout strains were analyzed for Vac8p functions including vacuole inheritance, Cvt pathway, localization of Nvj1p to the nucleus–vacuole junction, vacuole–vacuole fusion, as measured by vacuole morphology, and sensitivity to 0.2% caffeine (Table 2). Each potential Vac8p-binding partner with an identifiable Vac8p function appeared to be primarily required for only one Vac8p-related process (Table 2). Some of the candidate partners identified in the yeast two-hybrid test showed no known phenotypic relationship to Vac8p. These may not function with Vac8p, but rather may function with the other yeast ARM repeat protein, Srp1p (27).

**Vac8p localizes to discrete domains on the vacuole membrane**

In wild-type cells, Vac8p is enriched in at least two types of patches on the vacuole membrane. Vac8p accumulates at nucleol–vacuole junctions, an extended interface between the outer membrane of the nucleus and the vacuole (4,20), and at vacuole–vacuole junctions, sites of homotypic vacuole fusion (28) (Figure 2). Notably, Vac17p is not required for the accumulation of Vac8p in either of these regions (Figure 2). These observations suggest that Vac8p may localize to specific regions of the vacuole membrane, each of which is dedicated to a single function.

To test this hypothesis further, we asked whether Vac8p accumulates in a third region on the vacuole that contains Vac17p. Vac17p is found in a few small spots on the vacuole membrane (2), but the levels of endogenous Vac17p are too low to accurately test the degree of Vac8p colocalization with Vac17p. Therefore, we used a stabilized vac17 mutant, vac17ΔPEST, whose protein levels are ~10-fold higher than those of wild type. Vac17p-ΔPEST accumulates in large patches on the vacuole membrane in regions near the bud tip or the mother-bud neck (2). Notably, in the presence of Vac17p-ΔPEST-GFP, there was a significant redistribution of Vac8p-monomeric red fluorescent protein (mRFP) to the regions of the vacuole membrane at the bud tip of small-budded cells and at the mother-bud neck in large-budded cells, the same sites where Vac17p-ΔPEST-GFP accumulates (Figure 2). These results suggest that in wild-type cells, a portion of Vac8p colocalizes with Vac17p on the vacuole in a third region that is distinct from the nucleus–vacuole junction or the vacuole–vacuole junction.

**Each Vac8p function requires a subset of ARM repeats**

The observations that each Vac8p-binding partner has a unique function and that Vac8p localizes to at least three
distinct regions on the vacuole suggest that Vac8p-mediated coordination of multiple vacuole functions may be analogous to the orchestration of multiple cellular processes by β-catenin. The multiple binding partners of β-catenin compete with each other for access to specific ARM repeats. Thus, we generated a series of vac8 mutants each missing a single ARM repeat. Each mutant was analyzed for all known Vac8p functions (Figure 3A). In addition, using a yeast two-hybrid test, the interactions between each mutant with known Vac8p-binding partners were examined (Figure 3B).

These studies showed that ARM2 and ARM5 were required for each Vac8p function and for Vac8p interaction with each binding partner. In addition, each function also required a set of additional repeats that varied for each individual function. For example, vacuole inheritance required the least ARM repeats; in addition to ARM2 and ARM5, deletion of ARM6 partially blocked vacuole inheritance. In contrast, proper localization of Nvj1p to the nucleus–vacuole junction required ARM2–6 and ARM10–11 (Figure 3A). The results obtained in the functional assay largely paralleled the results of the ability of the ARM deletion mutants to bind each partner.

The total cellular levels of each mutant Vac8p were similar to those of wild-type Vac8p (Figure 3C). In addition, all mutant proteins localized to the vacuole membrane (Figure 3D). However, Vac8p-ΔARM1, ΔARM2 and ΔARM3 had lower levels of protein on the vacuole membrane; an antibody sandwich technique was required to visualize Vac8p in these three mutants (Figure 3D).

The lower levels of Vac8p in the vac8-ΔARM1, ΔARM2 and ΔARM3 mutants are unlikely to account for the loss of specific Vac8p functions; the levels of Vac8p-ΔARM1 and ΔARM2 were similar, yet Vac8p-ΔARM1 functioned similarly to wild-type Vac8p. That the levels of vacuole-membrane-associated Vac8p-ΔARM1, ΔARM2 or ΔARM3 are lower than those of wild type suggests that Vac8p association with the vacuole membrane requires the first three ARM repeats. While acylation of Vac8p is the major means by which Vac8p associates with the vacuole membrane, in the absence of acylation, approximately 10–15% of the Vac8p remains on the vacuole (7) presumably because of interaction between Vac8p and some unknown vacuolar membrane protein(s).

Vac8p-binding partners may compete with each other for access to Vac8p

The observation that interactions between Vac8p and its binding partners require an overlapping subset of the ARM repeats (ARM2 and ARM5) raised the possibility that the Vac8p-binding proteins compete with each other for access to Vac8p. Further support that competition occurs is that a 10-fold increase in Vac17p (Vac17p-ΔPEST-GFP) redistributed Vac8p-ΔRFP from the nucleus–vacuole junction and the vacuole–vacuole junction to a ‘Vac17p’-containing domain (Figure 2).

Competition for access to Vac8p would be influenced both by the steady-state levels of each binding partner and by the relative affinities for Vac8p of each binding partner. We do not currently have an in vitro assay to measure relative affinities, instead we analyzed the levels of each partner. The intracellular steady-state levels of Vac8p are higher than the levels of all the Vac8p-binding partners. Recent genome-wide studies of intracellular protein levels estimated that there are 5700 Vac8p molecules, 1900 Nvj1p molecules, 500 Vac17p molecules, 243 Tco89p molecules...
Figure 3: ARM2 and ARM5 of Vac8p are required for all Vac8p functions tested. A) Functional analysis of Vac8p mutants missing a single ARM repeat. Each Vac8p mutant was transformed into a vac8Δ strain and analyzed for vacuole inheritance (Inheritance), vacuole–vacuole fusion (Fusion), resistance to caffeine (Caffeine), the cytoplasm-to-vacuole protein targeting pathway (Cvt) and the proper localization of Nvj1p to the nucleus–vacuole junction (NVJ). Required: >85% of cells have mutant phenotypes; partially required: 35–85% of cells have mutant phenotypes; not required: <35% of cells have mutant phenotypes. B) ARM2 and ARM5 are required for Vac8p interaction with its binding partners. Full-length Vac8p (WT) or Vac8p missing one ARM repeat as indicated was fused with the Gal4p-binding domain in one plasmid (Tryptophan, TRP, marker). The same fragments listed in Table 3 and full-length of NVJ1 were fused with the Gal4p-activation domain (Leucine, LEU, marker). Both the Gal4p-binding domain and Gal4p-activation domain constructs were introduced into PJ69-4A and patched on SC-LEU-TRP-HIS-ADE plates containing 30 mM 3-amino-triazole, and grown at 24°C for 4 days. *TCO89 and NVJ1 plates were grown for 8 and 12 days, respectively. C) Deletion of a single ARM repeat does not affect cellular Vac8p levels. Crude extracts were analyzed for Vac8p levels. The same membrane was also probed with anti-Pgk1p antibodies. D) ARMs 1–3 play a role in Vac8p localization. The vacuole membrane (red) was visualized with mouse anti-Vma2p and rhodamine-red-conjugated donkey anti-mouse IgG. Vac8p (green) was labeled with rabbit anti-Vac8p and Alexa-488-conjugated goat anti-rabbit IgG. For Δarm1, Δarm2 and Δarm3, Vac8p was labeled with rabbit anti-Vac8p, goat anti-rabbit IgG and Alexa-488-conjugated donkey anti-goat IgG. Scale bar is 5 μm.
and 150 Apg13p molecules per cell (23,29). We used HA (influenza hemagglutinin)-tagged functional proteins to further estimate the relative levels of Vac8p and Vac17p on the vacuole, and observed that the vacuolar level of Vac8p is about 300-fold higher than that of Vac17p (Figure 4).

That Vac8p levels are higher than that of each of its binding partners suggests that not all the cellular Vac8p is bound to a partner protein. Thus, there is unlikely to be global competition for access to Vac8p. However, the fact that an increased level of Vac17p causes redistribution of Vac8p suggests that competition for binding to Vac8p occurs.

**Low levels of Vac8p preferentially support vacuole inheritance**

In order to further test for potential competition for access to Vac8p, we lowered the steady-state levels of Vac8p 10-fold via expression of VAC8 from the VAC17 promoter (Figure 5A). This level of Vac8p is still higher than endogenous Vac17p levels, most likely because unlike Vac17p, Vac8p does not contain known rapid degradation signals. The low levels of Vac8p did not affect its localization (Figure 5B,C).

Lowering Vac8p levels 10-fold affected a subset of Vac8p processes. For example, it resulted in a dramatic loss of Nvj1p-GFP concentration at the nucleus–vacuole junction (Figure 5D,E). Moreover, 10-fold less Vac8p resulted in partial caffeine sensitivity (Figure 5F). However, there was very little effect on vacuole inheritance and vacuole–vacuole fusion (Figure 5E). This lack of effect is likely due to the fact that very few Vac8p molecules are required to achieve normal vacuole inheritance and vacuole–vacuole fusion. In case of vacuole inheritance, it is also possible that the relative affinity of Vac17p for Vac8p may be high. Thus, when Vac8p levels are reduced 10-fold, vacuole inheritance is preferentially maintained. It appears that there is a correlation between relative levels of each Vac8p-binding protein and the effect of lowered Vac8p on the corresponding functions.

**Discussion**

Five new Vac8p-binding partners were identified in this study. Among them, Tco89p was found to be required for a novel Vac8p function: caffeine resistance. Tco89p is a component of the Tor complex 1 (21,30,31). Consistent with this function, a portion of Tco89p localizes at punctate clusters adjacent to the plasma membrane, the major site of the Tor complexes. However, immunogold electron microscopy showed that Tco89p is predominantly present on the vacuole membrane (21,32). This localization further supports the hypothesis that Tco89p associates with Vac8p. Moreover, like vac8 mutants, tco89Δ is hypersensitive to caffeine (21).

Our results indicate that Vac8p interacts with multiple proteins, each dedicated to a distinct function. There are at least three places on the vacuole membrane where Vac8p concentrates, each involved in a single Vac8p-related function. These observations raise the question of whether the diverse functions of Vac8p are coordinated with each other. As described further, we postulate that in rapidly dividing cells, organelle inheritance and organellar functions occur simultaneously, yet are spatially segregated.

The best known example of the separation of organelle inheritance from organellar function is the temporal separation of Golgi function from Golgi inheritance observed in vertebrate cells (33,34). At the onset of mitosis, the Golgi divides and Golgi function temporarily ceases. Vertebrate cells are predominantly in interphase and spend a small fraction of their time in mitosis. Thus, a transient block in Golgi function may not have a large effect on cellular physiology.

In a rich environment, yeast cells rapidly divide and thus most of their life cycle is dedicated to organelle inheritance and cell division (35–37). For yeast cells and higher eukaryotic cells that undergo rapid division, a temporary cessation of organelle function may be detrimental. Indeed, yeast organelles likely remain functional during their inheritance. Note that vacuole inheritance, the formation of a nucleus–vacuole junction and vacuole–vacuole fusion all occur simultaneously within a single cell (38).

There may be novel mechanisms to enable organelle inheritance and organellar functions to occur at the same time. Our findings that Vac8p, a member of the myosin V vacuole transport complex, is required both for vacuole inheritance and several additional vacuole-related processes suggest that Vac8p plays a pivotal role in coordinating vacuole inheritance with other vacuolar functions.

In further support that Vac8p plays a role in the coordination of multiple processes, it appears that Vac8p partners...
compete with each other for access to Vac8p. First, increasing Vac17p levels recruits Vac8p to a new location on the vacuole membrane (Figure 2). Second, lowering the steady-state levels of Vac8p differentially affects Vac8p functions and has the least effect on vacuole inheritance and homotypic vacuole–vacuole fusion (Figure 5). Third, overexpression of Nvj1p partially inhibits vacuole inheritance (4). That the binding partners compete for access to Vac8p is consistent with the finding that ARM2 and ARM5 are required for all Vac8p-related functions tested (Figure 3).

Figure 5: A 10-fold reduction in Vac8p levels has distinct consequences for each Vac8p function. A) Expression of VAC8 under the control of VAC17 promoter results in a 10-fold reduction in Vac8p levels. vac8Δ cells carrying vector (pRS416), wild-type VAC8 or P_{VAC17}-VAC8 plasmid were subjected to immunoblot analysis. 1 x indicates 0.2 OD_{600} cells. To compare the amounts of sample loaded, the same membrane was also probed with anti-Vph1p antibodies. B, C) P_{VAC17}-Vac8p is associated with the vacuole membrane. The vacuole membrane (red) and Vac8p (green) were visualized as before. In B, Vac8p was visualized with rabbit anti-Vac8p antibodies and Alexa-488-conjugated goat anti-rabbit IgG. In C, *Vac8p (green) was visualized with rabbit anti-Vac8p antibodies, goat anti-rabbit IgG and Alexa-488-conjugated donkey anti-goat IgG. Scale bar is 5 μm. D, E) P_{VAC17}-Vac8p supports vacuole inheritance and homotypic vacuole fusion but does not maintain proper localization of Nvj1p. Inheritance: vacuole inheritance. Fusion: vacuole morphology was used to assess vacuole fusion. Cells with less than six vacuole lobes were counted as wild type. The ratio of wild-type cells was reported. NVJ: nucleus–vacuole junction formation was reported as described in Figure 1B. Scale bar is 5 μm. F) P_{VAC17}-Vac8p partially restores caffeine resistance. vac8Δ cells carrying the indicated plasmids were spotted onto SC-URA (Uracil) and SC-URA +0.2% caffeine plates, and incubated at 24°C for 3 and 5 days, respectively.
We propose that Vac8p binds each partner at the correct place on the vacuole membrane to ensure that a single function occurs at a unique membrane subdomain. Binding of Vac17p to Vac8p on the segregation structure may prevent the binding of other Vac8p partners and thus dedicate that region to vacuole membrane movement. Similarly, the complex between the as yet unidentified fusion partner(s) and Vac8p may reside at the vacuole–vacuole junction and function solely for vacuole–vacuole fusion. Moreover, it is likely that binding of Nvj1p to Vac8p at the nucleus–vacuole junction excludes other Vac8p-binding partners. During vacuole inheritance, the nucleus–vacuole junction remains in the mother cell and does not move into the bud (our unpublished observations).

The biogenesis and inheritance of the vacuole yeast has several similarities with the biogenesis and inheritance of other yeast organelles including the late Golgi (39), the cortical endoplasmic reticulum (ER) (40) and peroxisomes (41). Each of these organelles likely functions throughout the cell cycle. In addition, in the absence of inheritance, each organelle appears to have alternative pathways to generate an organelle in the bud (42–44). Notably, the inheritance of each organelle requires the interaction of a class V myosin, Myo2p for most organelles and Myo4p for the cortical ER (45). The myosin V motors likely attach to each organelle via organelle-specific receptors. Based on our observations of vacuole inheritance, we postulate that a component(s) of these receptors may have multiple functions, and like Vac8p, may serve to spatially coordinate organelle inheritance. Moreover, we predict that the spatial separation of organelle inheritance and other organelar functions may be a common occurrence in rapidly dividing cells.

Materials and Methods

Yeast strains and plasmids

Yeast strains used in this study are listed in Table 3. A mRFP tag (47) was integrated chromosomally to generate VAC8-mRFP as described (48).

vac8ΔARMs 1-3 were constructed using the Transformer™ Site-Directed Mutagenesis Kit (ClonTech, Mountain View, CA, USA). vac8ΔARMs 4-8 were constructed by the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). vac8ΔARMs 9-11 were described previously (7).

pVAC8::VAC8 was constructed by in vivo recombination in LWY2887. The VAC8 ORF, amplified with V17promV8 (GATAAGAAACAGCTCGCATAAG- GAAACA AGGACA CATCGAT TAATGGGT TCATGTTGTAGTTGC) and V8n17UTR (CGAAT AAACATTTGG AGCAAAAGAAGTAGTTAGG- CAGCATGCTGGGACATTACGCGGACCTGCGATTTG) from pEYGFP2. This fragment was cotransformed into LWY7235 with SacI/Nof-digested pNVJ1. Plasmids from transformants exhibiting a GFP signal were recovered and sequenced.

At least two distinct alleles of Vac17p are commonly found in Saccharomyces cerevisiae

During the course of these studies, we found that the VAC17 gene (GenBank AY532640) from LWY7235 had 25 nucleotide differences when compared with the VAC17 gene isolated from the strains BY4741 or S288c (Saccharomyces Genome Database http://www.yeastgenome.org/). These differences include 11 nucleotides in the ORF that result in seven amino acid changes. They are S74N, P116S, T210A, T255P and S330P, and an insertion of two amino acid residues, Leu and Asn, after residue 393. Most of these differences are also found in the VAC17 genes of other closely related species of yeast (see http://db.yeastgenome.org/cgi-bin/FUNGI/ nph-showAlign?locus=YCL0633Y for a detailed alignment). We tested for functional differences between these alleles, but none were observed.

Immunofluorescence labeling and microscopy

Yeast vacuoles were labeled with FM4-64 (Molecular Probes, Eugene, OR, USA) as described (7). Cells were observed using an Axioscope 2 (Carl Zeiss Microlmaging, Jena, Germany) or the DeltaVision RT Restoration Microscopy System (Applied Precision, Issaquah, WA, USA).

Indirect immunofluorescence was performed to visualize Vac8p. Spheroplasts were prepared as described (2). For cells with Vac8p levels similar to wild type, Vac8p was labeled with rabbit anti-Vac8p (1:100) and Alexa-488 conjugated goat anti-rabbit IgG (1:200). For cells with low levels of Vac8p, spheroplasts were treated with a “sandwich” of rabbit anti-Vac8p (1:100), goat anti-rabbit IgG (1:240), and Alexa-488 conjugated donkey anti-goat IgG (1:200). Vascular membranes were visualized with mouse monoclonal anti-vacuolar ATPase (60 kD subunit) (1:100) (Molecular Probes) and rhodamine-red-conjugated donkey anti-mouse IgG (1:200) (Jackson Immuno Research, West Grove, PA, USA). Images were obtained with a laser scanning confocal system (MRC-1024, Bio-Rad, Hercules, CA, USA) as described (7). A single slice of a Z series is shown for each sample.

Immunoblot analysis

Total cell extracts were prepared as described (7). Briefly, five OD600 units of exponentially growing cells (OD600 = 0.2–0.5) were lysed by vigorous vortexing with 0.5 mm glass beads in 50 μl of sorbitol lysis buffer (0.3 M sorbitol, 10 mM Tris, pH 7.5, 0.1 M NaCl, 1 mM MgCl2, 1.0 mM ethylene-diaminetetraacetic acid and 1 × Sigma protease inhibitor cocktail) for 2 min at 4°C. Extracts were mixed with 17 μl of 4 × SDS loading buffer, heated at 80°C for 10 min and then centrifuged at 20 800 g for 5 min. Ten to 30 μl

Traffic 2006; 7: 1368–1377

1375

Table 3: Strains used in this study

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was run on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes and subjected to immunoblot analysis for Vac8p, Pga1p or Ape1p.

**Comparison of vacuole-membrane-associated Vac17p and Vac8p levels**

To compare relative levels of Vac17p and Vac8p, we constructed p6XHA-VAC8 and p3XHA-VAC17, which support vacuole inheritance to 90 and 70%, respectively. Vacuoles were isolated from vac8pΔ (p6XHA-VAC8) and vac17AΔ (p3XHA-VAC17), and the proteins were separated on a 5–10% gradient SDS-PAGE. After immunoblotting, the relative intensity of the HA epitope in each lane was measured using Scion Image™ software. Figure 4 shows a representative blot. The doublet bands may result from partial degradation or posttranslational modification. These bands were absent in vacuoles without HA-tagged proteins. The relative intensities of the doublet bands were measured (Figure 4), and the relative level of [Vac8p]/[Vac17p] was calculated with the following formula:

\[
\frac{[\text{Vac8p}]}{[\text{Vac17p}]} = \frac{\text{475(intensity of Vac8p)}}{\text{1156(intensity of Vac17p)}} \times \frac{15000 \text{ ng (Vac17p)}}{10 \text{ ng (Vac8p)}} \approx 300.
\]

Results presented are the average from three experiments. For each experiment, bands of similar intensities were used to ensure that the measured intensities were within the linear range of the enhanced chemiluminescence reagent.

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**References**


