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Homotypic Vacuole Fusion in Yeast Requires Organelle Acidification and Not the V-ATPase Membrane Domain

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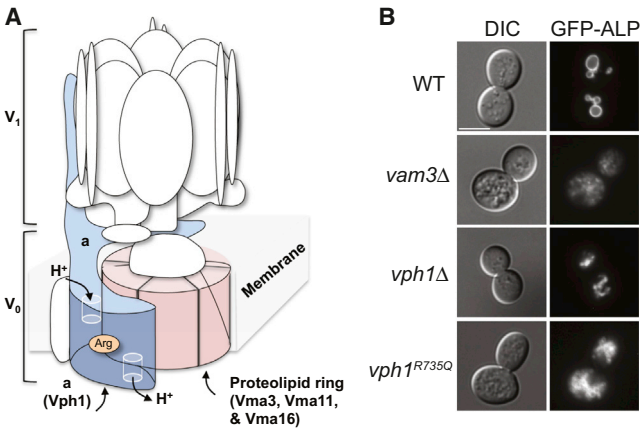
SUMMARY

Studies of homotypic vacuole-vacuole fusion in the yeast *Saccharomyces cerevisiae* have been instrumental in determining the cellular machinery required for eukaryotic membrane fusion and have implicated the vacuolar H^+-ATPase (V-ATPase). The V-ATPase is a multisubunit, rotary proton pump whose precise role in homotypic fusion is controversial. Models formulated from in vitro studies suggest that it is the proteolipid proton-translocating pore of the V-ATPase that functions in fusion, with further studies in worms, flies, zebrafish, and mice appearing to support this model. We present two in vivo assays and use a mutant V-ATPase subunit to establish that it is the H^+-translocation/vacuole acidification function, rather than the physical presence of the V-ATPase, that promotes homotypic vacuole fusion in yeast. Furthermore, we show that acidification of the yeast vacuole in the absence of the V-ATPase rescues vacuole-fusion defects. Our results clarify the in vivo requirements of acidification for membrane fusion.

INTRODUCTION

Membrane-fusion events are tightly regulated in all eukaryotic cells through mechanisms conserved across evolution. Homotypic fusion of vacuole membranes in the yeast *Saccharomyces cerevisiae* has been extensively studied as a model system to understand this process. The original in vitro assays were based on incubation of purified vacuoles isolated from two different yeast strains: one lacking alkaline phosphatase (ALP), the other lacking the vacuolar-processing enzyme Pep4p. Vacuolar fusion results in content mixing of the two populations and activation of the enzymatic activity of ALP via processing by Pep4p, which is measured by a colorimetric assay (Conradt et al., 1994; Haas et al., 1994). Such studies have demonstrated many requirements for vacuolar membrane fusion, including a requirement for SNARE proteins (Nichols et al., 1997; Wickner, 2010) and the vacuolar H^+-ATPase (V-ATPase) (Baars et al., 2007; Bayer et al., 2003; Peters et al., 2001). The V-ATPase is a multisubunit proton pump (Figure 1A), and initial models suggested that the integral membrane subcomplex V_0, which is responsible for translocating protons, is required on both membranes for the final membrane-fusion reaction, possibly by acting as a fusion pore (Baars et al., 2007; Strasser et al., 2011; Stroupe et al., 2009). Models for such a physical role for V_0 in membrane fusion based on results from yeast led to a paradigm shift, resulting in the interpretation of data from worm, fly, zebrafish, and mouse studies to implicate direct V_0 involvement (Scott et al., 2011). Despite extensive literature on this topic, the role of the V-ATPase in yeast vacuole-vacuole fusion remains controversial for several reasons:

(1) It has been suggested that vacuole-vacuole fusion requires vacuolar acidification (Ungermann et al., 1999), making it difficult to distinguish the requirement for V-ATPase proton pumping from its physical presence on vacuole membranes for membrane fusion. Data demonstrating that vacuoles lacking V_0 do not fuse with each other (Baars et al., 2007; Bayer et al., 2003; Takeda et al., 2008) are difficult to interpret because the vacuole is not acidified, and it lacks an assembled V_0. Recently, point mutations in the proteolipid V_0 subunits (Vma3p, Vma11p, and Vma16p; see Figure 1A) have been generated that inhibit vacuole fusion 30%–90%, leaving some acidification activity intact (Strasser et al., 2011). However, even these mutants have at least a 30%–40% reduction in proton-pumping activity, and it remains difficult to separate the two functions.
(2) In vitro data addressing the requirement for V_0 subunits in vacuole-vacuole fusion are contradictory. The requirement of both partners in the fusion reaction to contain the V_0 subunit Vph1p is used as evidence that the physical presence of V_0 is necessary on both membranes for fusion (Baars et al., 2007; Bayer et al., 2003). However,



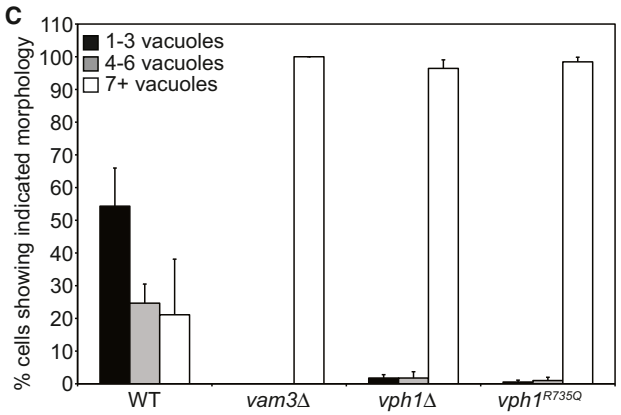


Figure 1. Acidification of the Vacuole Is Required for Vacuole Morphology Maintenance In Vivo

(A) The V-ATPase is composed of a V_1 ATP hydrolytic domain and a V_0 membrane domain. The V_0 domain is involved in H^+ translocation and contains subunit a and the proteolipid ring composed of three different subunits in yeast. The highly conserved Arg at position 735 of subunit a is required for V-ATPase H^+ translocation.

(B) Parent (WT; KEBY136), *vam3Δ* (ECY157), *vph1Δ* (ECY145), and *vph1*^{R735Q} (ECY149) cells expressing GFP-ALP (pLG230) as a vacuole maker were visualized by fluorescent (right column) or differential interference contrast (DIC) microscopy (left column). Images shown are from a 0.5 μm z stack. Scale bar, 5 μm.

(C) Quantification of the experiment shown in (A) (n = 900 cells/strain) is presented. SD is indicated.

See also Figure S1.

using the same assay and protocol (including the same parent yeast strains), we find that vacuoles lacking Vph1p fuse with Vph1p-containing vacuoles (see Figure S1A available online). Furthermore, vacuoles lacking the V_0 subunit Vma6p have only a modest inhibition of fusion with Vma6p-containing vacuoles (Baars et al., 2007; Takeda et al., 2008), and vacuoles lacking proteolipid subunits Vma3p or Vma16p fuse with vacuoles containing intact V-ATPase (Takeda et al., 2008) (Figure S1A).

(3) In vitro reconstitution experiments show that vacuolar SNAREs, Sec17p, Sec18p, the HOPS complex, ATP hydrolysis, and GTP-bound Ypt7p are sufficient for membrane fusion. This implies that components of the V-ATPase are not required for membrane fusion, at least in this reconstituted system (Stroupe et al., 2009).

In this report, we demonstrate the absolute requirement of acidification in homotypic vacuole fusion by introducing two assays that assess fusion in vivo. Using these assays in combination with a point mutation that results in a fully assembled and properly localized V-ATPase that does not pump protons, we show that vacuole acidification is required for homotypic vacuole fusion. Importantly, we use the exogenous vacuole-targeted H^+-translocating inorganic pyrophosphatase from *Arabidopsis thaliana*, *AVP1*, to demonstrate that vacuole-fusion defects can be rescued by acidification of this organelle.

RESULTS

We set out to determine whether vacuole-vacuole fusion requires the acidification function of the V-ATPase or the physical presence of V_0. Vacuolar morphology in *S. cerevisiae* can be a measure of the balance between vacuole fusion and fission because strains with fusion defects have highly fragmented vacuoles (Baars et al., 2007; Strasser et al., 2011; Wada et al., 1992). In vitro assays have established the requirement of the SNAREs Vam3p and Nyv1p for homotypic vacuole fusion (Nichols et al., 1997), and yeast cells lacking Vam3p (*vam3Δ*) exhibit highly fragmented vacuoles using the vacuolar membrane marker GFP-ALP. ALP is a reliable vacuole membrane marker because, unlike the dye FM4-64, ALP transport to the vacuole is not affected by defects in vacuole acidification (Graham et al., 2003; Perzov et al., 2002). *vam3Δ* yeast cells have numerous, small vacuolar vesicles (100% of cells display seven or more vacuoles), compared to the parent strain where the majority of cells have one to three vacuoles (Figures 1B and 1C) (Baars et al., 2007; Darsow et al., 1997; Nichols et al., 1997). We examined vacuole morphology in strains lacking the V_0 subunit Vph1p, and similar to the *vam3Δ* strain, 96% of *vph1Δ* cells have seven or more vacuoles, suggesting a possible vacuole-fusion defect (Figures 1B and 1C; Baars et al., 2007). To distinguish the acidification function of the V-ATPase from the physical presence of the V_0 proteolipid channel on vacuoles, we replaced the genomic copy of *VPH1* with a mutant version, *vph1*^{R735Q}, which allows proper assembly and trafficking of the V-ATPase to the vacuole but completely abrogates ATP hydrolysis and proton pumping into this organelle (Kawasaki-Nishi et al., 2001). To confirm the phenotype of *vph1*^{R735Q} cells, we found that Vph1p^{R735Q} was localized to the vacuole (Figure S1B), and cells expressing *vph1*^{R735Q} showed growth defects on media containing $ZnCl_2$ or $CaCl_2$, demonstrating that, as expected, the assembled V-ATPase complexes containing Vph1p^{R735Q} are not functional (Figure S1C; Finnigan et al., 2012). Acidification of yeast vacuoles by the V-ATPase is required to drive the sequestration of potentially toxic levels of metal ions such as Zn^{2+} and Ca^{2+}, and therefore, a growth defect on this media is a measure of nonacidified vacuoles. As with *vam3Δ* and *vph1Δ* strains, 98% of *vph1*^{R735Q} cells have seven or more vacuoles (Figure 1C). Our results are not the consequence of strain-specific effects because BY4741 and BJ3505 strains with the *vph1*^{R735Q} mutation also exhibited highly fragmented vacuoles (Figure S1D). These data indicate that it is

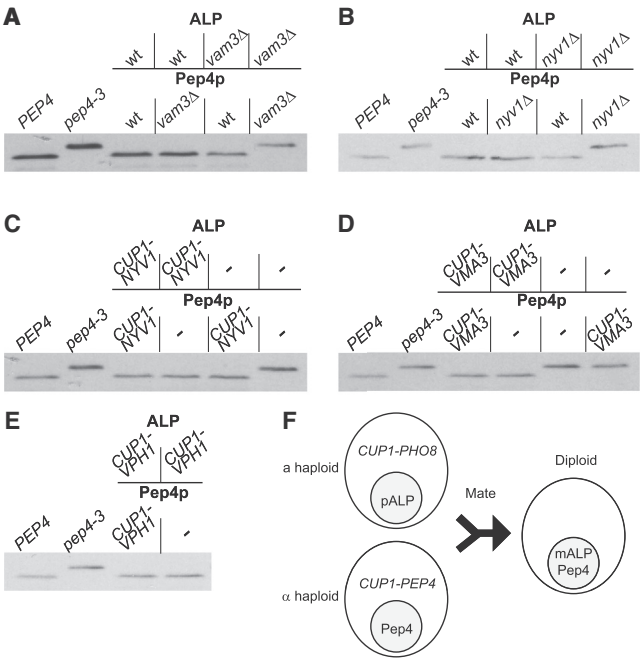


Figure 2. V_0-Dependent Proton Pumping Is Required on Only One Vacuole for Homotypic Fusion In Vivo, as Assessed using an ALP-Processing Assay

(A–E) Immunoblot analysis was used to assess whether ALP was present in its pro (pALP) or mALP form in lysates of diploids formed using the assay shown in (F). Lysates of *PEP4* (RPY10) and *pep4-3* (SF838-9Da) haploid cells were included in the analysis to indicate the positions of mALP and pALP. In (A) and (B), the strains used are indicated. In (C), *nyv1*Δ strains (KEBY189 and KEBY192) were used, and the presence or absence of the *CUP1-NYV1* plasmid (pTC5) is indicated. Similarly, in (D) and (E), the strains used were *vma3*Δ (KEBY169 and KEBY171) and *vph1*Δ (ECY145 and ECY147), respectively, and the plasmids present were *CUP1-VMA3* (pTC6) or *CUP1-VPH1* (pGF669), as indicated.

(F) Schematic representation of the ALP-processing assay to identify requirements of homotypic vacuole fusion in vivo.

See also Figure S2.

the acidification function of the V-ATPase, rather than the physical presence of the V_0 subcomplex, that is required for homotypic vacuole-vacuole membrane fusion in vivo.

Acidification Function of the V-ATPase Promotes Vacuole-Vacuole Fusion

To explore further the role of the V-ATPase in membrane fusion in vivo, we developed two assays, both of which take advantage of the natural fusion of vacuoles from parent cells that occurs in the newly formed zygote during the first cell division following yeast mating (Weisman and Wickner, 1988). In the first assay, we used ALP (*PHO8*) and Pep4p, the protease that initiates ALP cleavage once it reaches the vacuole, to test in vivo vacuole fusion. This assay is based on the conventional content-mixing assay, but results are generated in vivo by Pep4p-dependent cleavage of ALP, as assessed by western blot (Figure 2). The yeast cells used for this assay have been engineered so that they do not express genomic copies of ALP (*pho8Δ*) or Pep4p (*pep4-3*) but produce one or the other from plasmids under the control of the copper-inducible *CUP1* promoter. This strategy ensures that vacuoles in one parental strain contain ALP, and vacuoles in the other strain contain Pep4p (Figure 2F). Growth on media containing copper and subsequent removal of the copper allowed a pulse of protein to be synthesized and then chased into the vacuole. Efficient expression and shutoff with the *CUP1* promoter are shown in Figure S2A. Both Pep4p and ALP are chased to the vacuole without additional protein production, ensuring that ALP processing (conversion of pro-ALP [pALP] to mature ALP [mALP]) is a measure of vacuole-vacuole fusion and not, for example, endosome-vacuole fusion. The latter scenario is rendered even more unlikely because Pep4p and ALP take different transport routes from the Golgi to the vacuole (Cowles et al., 1997; Piper et al., 1997) (Figure S2B). Cells of opposite mating types, expressing either ALP or Pep4p in the vacuole, were shifted to media lacking copper for 24 hr, mated, and diploids selected on plates lacking copper. For parent strains crossed with each other (wild-type [WT] × WT), we observed full processing of pALP to its mature form after diploid formation, indicating vacuole-vacuole fusion (Figure 2A).

We also tested strains lacking the vacuolar SNAREs Vam3p or Nyv1p. We found that *vam3Δ* or *nyv1Δ* strains crossed with the parent strain produced mALP, indicating vacuole-vacuole fusion. No mALP was produced in crosses of either *vam3Δ* × *vam3Δ* or *nyv1Δ* × *nyv1Δ*, indicating a defect in homotypic vacuole fusion in vivo (Figures 2A and 2B). This is consistent with results from in vitro experiments showing the requirement for vacuolar SNAREs on only one membrane for vacuole-vacuole fusion (Nichols et al., 1997). To ensure that our results were not complicated by new synthesis of protein following diploid formation, we repeated the experiment using *CUP1-NYV1*. Removal of copper from the medium during the assay ensured that no new synthesis of Nyv1p, like ALP and Pep4p, occurred. As shown in Figure 2C, the results using *CUP1-NYV1* were identical to those using the deletion strains, once again validating our in vivo content-mixing assay for vacuole fusion. All further experiments using this assay were done with *CUP1* expression/shutoff of the gene of interest.

We then used our assay to determine the in vivo requirement for the V_0 in homotypic vacuole fusion. As shown in Figure 2D, the V_0 subunit Vma3p on one membrane is sufficient for processing of pALP, which is indicative of homotypic vacuole fusion. However, ALP is not processed if it is expressed in the *vma3Δ* strain, possibly due to the requirement of an acidified environment to prime ALP for protease cleavage.

We also looked at the requirement of the V_0 subunit Vph1p in vacuole-vacuole fusion in our in vivo content-mixing assay. Vph1p present on only one membrane was sufficient for ALP processing to its mature form, again clearly demonstrating in vivo that fusion can occur if the V_0 subcomplex is present on one membrane only, in agreement with in vitro results (Figures 2E and S1A; Takeda et al., 2008).

To substantiate and extend the results of our in vivo content-mixing assay, we developed a second assay to measure vacuole-vacuole fusion in vivo, this time using live-cell imaging. This microscopic assay analyzes overlap of fluorescent vacuole markers in the zygotic bud following mating. Two parental strains were transformed with different fluorescent versions of ALP: one tagged with GFP, and the other with mCherry. Vacuoles in the large zygotic bud were scored for overlap of red and green

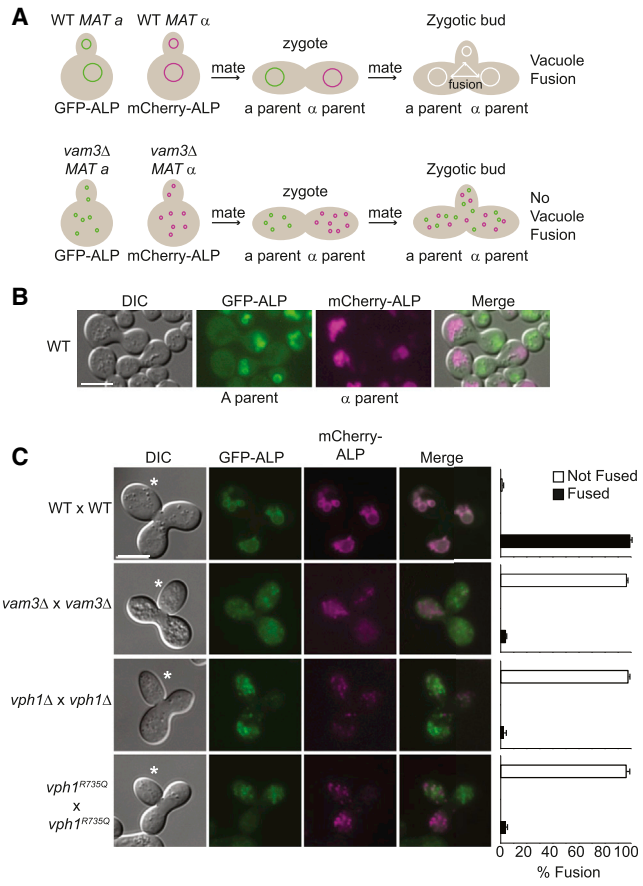


Figure 3. Vacuole Acidification Is Required for Fusion In Vivo, as Assessed with a Microscopic Assay

(A) Schematic representation of a microscopic zygotic bud assay to identify requirements of homotypic vacuole fusion in vivo. In this schematic, fused vacuoles are depicted in white, and unfused vacuoles are depicted in green or red.

(B) Haploid parent cells expressing either GFP-ALP (pLG230) or mCherry-ALP (pMP2) were visualized by fluorescence or DIC microscopy during mating at the shmoo stage. Scale bar, 5 μm.

(C) Haploid cells expressing either GFP-ALP or mCherry-ALP were mated in YEPD and visualized by fluorescence and DIC microscopy. Large budded zygotes are shown for WT × WT (ECY153 and ECY155), *vam3Δ* × *vam3Δ* (ECY157 and ECY158), *vph1Δ* × *vph1Δ* (ECY145 and ECY147), and $vph1^{R735Q}$ × $vph1^{R735Q}$ (ECY149 and ECY151). Buds are indicated by an asterisk. Scale bar, 5 μm. Quantification of fusion in the zygotic bud in WT × WT (n = 90), *vam3Δ* × *vam3Δ* (n = 81), *vph1Δ* × *vph1Δ* (n = 40), and $vph1^{R735Q}$ × $vph1^{R735Q}$ (n = 51) is shown to the right of the cell images. Black bars indicate cells with fused vacuoles in the bud, and white bars indicate zygotic buds with vacuoles that are not fused. SD is indicated.

fluorophores (Figure 3A). Defects in vacuole fusion are reflected by numerous small fragmented vacuoles with nonoverlapping red and green fluorescent signals in the large zygotic bud as opposed to the WT-fused vacuoles and overlap of red and green fluorophores seen with fusion-competent vacuoles. To quantify our assay, vacuoles in the zygotic bud were scored as "fused" if the red and green signals overlapped by visual inspection and as "not fused" if there was no overlap of the two fluorophores. The total number of cells showing fused or unfused vacuoles divided by the total number of cells scored is expressed as percent (%) fusion (Figure 3C). To show that vacuole fusion did not occur until the zygotic bud formed, we observed cells in the early zygote-fusion stage expressing either GFP-ALP or mCherry-ALP and saw no overlap between the two fluorophores (Figure 3B).

Our zygotic bud-fusion assay confirmed that 99% of the zygotes from WT crosses had fused vacuoles (Figure 3C). In contrast, *vam3Δ* crosses produced highly fragmented vacuoles with only 4% of cells scored as fused, demonstrating an almost complete block in vacuole-vacuole fusion in the large zygotic bud. Similarly, *vph1Δ* crosses resulted in fragmented vacuoles with only 3% fusion scored. Crucially, only 4% of $vph1^{R735Q}$ zygotes were scored as fused, and the vacuoles were highly fragmented in the zygotic bud. This confirms our previous observations of steady-state fragmented vacuole morphology in $vph1^{R735Q}$ cells and substantiates the conclusion that it is the proton-pumping activity of Vph1p rather than the physical presence of V_0 that is required for vacuole-vacuole fusion.

Acidification by the Plant Pyrophosphatase in the Absence of V-ATPase Rescues Vacuole-Fusion Defects

We reasoned that if WT vacuole fusion requires acidification rather than the physical presence of the V-ATPase, then the fragmentation phenotype and fusion defects seen in *vph1* mutants should be rescued by V-ATPase-independent acidification of the vacuoles. To achieve this, we expressed the vacuole-targeted H^+-translocating inorganic pyrophosphatase from *Arabidopsis thaliana*, *AVP1*, in fusion-defective yeast. AVP1 protein is present on the vacuolar membrane of plant cells but is not found in other eukaryotes. It couples cytoplasmic pyrophosphate (PPi) hydrolysis to translocation of protons into the vacuolar compartment (Baltscheffsky et al., 1999; Sarafian et al., 1992). AVP1 protein expressed in yeast cells has been shown to localize to the vacuole membrane and lead to vacuole acidification, but only in the absence of the yeast cytoplasmic inorganic pyrophosphatase, Ipp1p, which otherwise hydrolyzes cytosolic PPi (Drake et al., 2010; Pérez-Castiñeira et al., 2011; Perez-Castineira et al., 2002). We examined vacuole morphology and vacuole pH in yeast expressing *AVP1* in place of the endogenous *IPP1*. Of the parental "wild type" cells, 84% had one to three vacuoles, and the vacuolar pH measured using the ratiometric, pH-sensitive dye BCECF was 5.67 (Figures 4A–4C). This was consistent with our previous vacuole morphology results (Figure 1) and with measurements of vacuolar pH by others (pH 5.5–5.9) (Diakov and Kane, 2010; Diakov et al., 2013; Martínez-Muñoz and Kane, 2008; Plant et al., 1999; Tarsio et al., 2011). As seen previously, *vph1Δ* cells displayed fragmented vacuoles, and as expected, 98% of double-mutant *vph1Δ ipp1Δ* cells expressing *IPP1* also displayed fragmented vacuoles (Figures 4A and 4B). The vacuolar pH in the *vph1Δ ipp1Δ* cells expressing *IPP1* rose to 6.62 (Figure 4C). *vph1Δ ipp1Δ* cells expressing both *IPP1* and *AVP1* had fully fragmented vacuoles (data not shown) because the Ipp1 protein very efficiently hydrolyzes the available cytosolic pyrophosphate (Sarafian et al., 1992). Importantly, *vph1Δ ipp1Δ* cells expressing only *AVP1* showed significant rescue of vacuole fragmentation, with 54% of these cells containing one to three vacuoles (Figures 4A and 4B). We measured a vacuolar pH of 6.42 in these cells (Figure 4C). Thus, expression of *AVP1* in cells lacking the

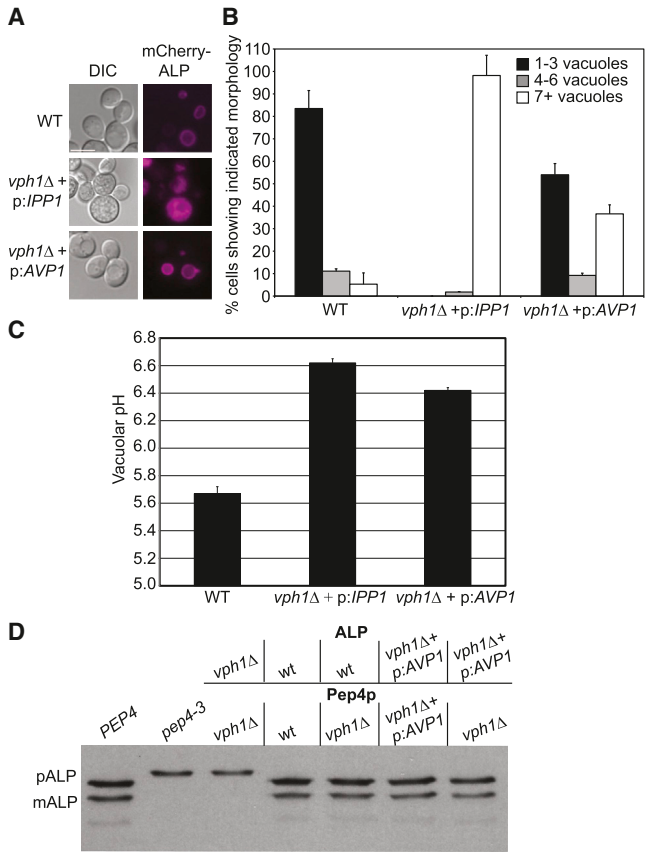


Figure 4. Expression of Vacuole-Localized *Arabidopsis thaliana* Pyrophosphatase *AVP1* Rescues the Vacuole-Fusion Defect in Cells Lacking Vph1p

(A) WT (SF838-1Dα), *vph1Δ* + p:*IPP1* (*vph1::hyg ipp1::kanMX*, ECY196 expressing plasmid-borne *IPP1* [p:*IPP1* and pEC139]), and *vph1Δ* + p:*AVP1* (*vph1::hyg ipp1::kanMX*, ECY196 with plasmid pLG362 [p:*AVP1*] swapped for plasmid pEC139 [see Supplemental Experimental Procedures] and expressing mCherry-ALP [pGF242]) were visualized using fluorescent and DIC microscopy. Scale bar, 5 μm.

(B) The vacuole morphology of WT (n = 170), *vph1Δ* + p:*IPP1* (ECY196, n = 213), and *vph1Δ* cells + p:*AVP1* (ECY196 with plasmid pLG362 swapped for plasmid pEC139, n = 636) expressing plasmid-borne mCherry-ALP (pGF242) was quantified. SD is indicated.

(C) Vacuolar pH was measured in WT (n = 4), *vph1Δ* + p:*IPP1* (ECY196, n = 5), and *vph1Δ* + p:*AVP1* (ECY196 with plasmid pLG362 swapped for plasmid pEC139, n = 5) using BCECF dye. SD is indicated.

(D) Lysates of *PEP4* (RPY10) and *pep4-3* (SF838-9Da) haploid cells were included in the analysis to indicate the positions of mALP and pALP in the in vivo ALP content-mixing assay. Processing of ALP is shown for *vph1Δ* diploids (ECY145 and ECY147), WT diploids (RYP10 and SF838-9Da), WT × *vph1Δ* diploids, *vph1Δ ipp1Δ* + p:*AVP1* diploids (LGY253 and LGY254), and *vph1Δ ipp1Δ* + p:*AVP1* × *vph1Δ* diploids. Plasmids present in ALP- or Pep4p-expressing cells in all lanes were pTC1 and pTC2.

V-ATPase (*vph1Δ* cells) decreases vacuolar pH from 6.62 to 6.42. The incomplete rescue of vacuole morphology and relatively modest decrease in vacuolar pH were very likely due to insufficient *AVP1* expression in some cells due to the variable copy number of the 2μ *AVP1* plasmid. These observations are consistent with the previously published ability of AVP1 protein to acidify yeast vacuoles only in the absence of Ipp1p (Pérez-Castiñeira et al., 2011) and suggest that sufficient expression of *AVP1* corrects the vacuole-fusion defect of *vph1Δ* cells. We then used our in vivo ALP content-mixing assay (Figure 2F) to confirm this interpretation. As shown previously, Vph1p is required on one membrane to promote vacuole fusion, as assessed by ALP processing (Figure 2). However, the expression of *AVP1* on one or both membranes in *vph1Δ ipp1Δ* cells results in clear ALP processing (Figure 4D), indicating that expression of *AVP1* can restore vacuole-vacuole fusion even in the complete absence of the V-ATPase. Together, these data demonstrate that acidification of yeast vacuoles in the absence of the V-ATPase is sufficient for promoting homotypic vacuole-vacuole fusion.

DISCUSSION

In vitro assays of yeast vacuole-vacuole fusion have provided tremendous insight into the general mechanisms of membrane fusion. To complement these studies, and to investigate the requirements for membrane fusion in vivo, we have developed two assays. Both of our assays are based on the premise that *S. cerevisiae* vacuoles fuse in vivo during the first cell division after zygote formation (Weisman and Wickner, 1988). Our first assay measures content mixing using biochemical protein processing, and the second relies on membrane mixing as defined by the overlap of fluorescent markers. Using these assays, we have investigated the controversial role of the V-ATPase complex in membrane fusion. In contrast to some previous in vitro reports, we found that the V_0 subcomplex of the V-ATPase is required on one membrane, not both, for vacuole-vacuole fusion in vivo. Furthermore, expression of a V-ATPase complex that was assembled and targeted to the vacuole but completely unable to translocate protons indicated that vacuole-vacuole fusion depends on the proton-translocation activity of the V-ATPase, and not its physical presence on the vacuole. To confirm this hypothesis, we expressed the plant pyrophosphatase *AVP1* in yeast to acidify vacuoles in the absence of the V-ATPase. Using cells that lacked the V-ATPase but expressed *AVP1*, we demonstrated that the acidification of at least one vacuole is the essential requirement for vacuole-vacuole fusion.

Our ability to lower the vacuolar pH of vp*h1Δ* cells and restore vacuolar morphology and membrane fusion suggests that vacuole acidification promotes vacuole-vacuole fusion. However, the question remains as to whether it is acidification per se, the change in proton motive force or membrane potential, or indeed a more indirect effect (e.g., translocation of a counter-ion) that is the key requirement for membrane fusion.

In light of our findings, it will be necessary to reevaluate data from other systems. For example, mutations in the mammalian V_0 sector subunit a genes (orthologous to yeast *VPH1*) led to membrane-fusion defects in synaptic vesicle exocytosis (Hiesinger et al., 2005) and phagocytosis (Peri and Nüsslein-Volhard, 2008), and these results were interpreted to implicate direct V_0 sector involvement, rather than defects in acidification. However, to convincingly separate the role of V_0 in acidification from other more-direct binding roles in membrane fusion, it will be necessary to prepare H^+-translocation dead but stable forms of the V-ATPase (Perzov et al., 2002). For example, generation of proton-translocation dead subunit a isoforms (homologous to

the Vph1p^{R735Q} mutant used in this study) would eliminate H^+ translocation without affecting assembly or targeting of the resulting V-ATPase. Generation of transgenic animals with corresponding mutations (e.g., *vha100-1* in *D. melanogaster*; Hiesinger et al., 2005) would more rigorously test whether it is the acidification function associated with the a1 subunit or rather the physical presence of its associated V_0 sector that is responsible for the observed phenotypes. Our findings have implications for the analysis of membrane fusion in all eukaryotic systems, and our assays pave the way for the assessment of factors required for vacuole membrane fusion in vivo. This will further our understanding of the fundamental process of membrane fusion.

EXPERIMENTAL PROCEDURES

In Vivo ALP-Processing Assay

Transformants were grown on selective minimal media plates (SD-Ura-Leu) containing 50 μM copper sulfate. Cells were switched to yeast extract peptone with dextrose (YEPD) (no copper) for 24 hr prior to setting up crosses. Diploids were selected on SD-Ade-Lys and grown for 24 hr at 30°C. Cells were then scraped off the selective plate and prepared for western blotting with antibodies to ALP.

Zygotic Bud-Fusion Assay

The zygotic bud-fusion assay was performed essentially as described for in vivo mitochondrial fusion (Mozdy et al., 2000). Live-cell images were acquired on a Zeiss Axioplan 2 fluorescence microscope with a 100× objective, and image processing was done using AxioVision (Zeiss) software. Images of mating cells were captured, and only large budded zygotes were quantified. Vacuoles were scored as fused if overlap between red and green signals was observed in the zygotic bud. Vacuoles were quantified as not fused if they remained fragmented in the zygotic bud, and the red and green signals had no appreciable overlap. Quantification is expressed as the percentage of total cells in each strain scored as fused or not fused and was performed in three or more independent experiments. SDs are shown. See additional method details in the Supplemental Experimental Procedures.

Live-Cell Imaging

Live-cell imaging of haploid cells in Figures 1 and S1 was performed by growing strains overnight in synthetic dextrose media to select for the plasmid. Cells were back-diluted in synthetic dextrose media to an OD_{600} of 0.5. Exponentially growing cells were collected by centrifugation at 3,000 rpm for 30 s and resuspended in 100 μl of water for imaging. Vacuole morphology in Figures 1 and S1 was quantified in 300 cells over 3 different days and categorized into one to three vacuoles, four to six vacuoles, and seven or more vacuoles, and SD is indicated. All live-cell images were acquired on a Zeiss Axioplan 2 fluorescence microscope with a 100× objective, and image processing was done using AxioVision software.

Rescue of Vacuole Morphology by *AVP*

Cells were prepared for imaging by overnight growth in synthetic dextrose media (-Leu-Ura) and dilution to an OD_{600} of 0.5 in YEPD plus ClonNat. Cells were grown to an OD_{600} of 1–1.5 and visualized using fluorescence microscopy. Quantification of vacuole morphology was performed by counting cells in each indicated category (one to three vacuoles, four to six vacuoles, and seven or more vacuoles) from images taken on 2 different days with Zeiss AxioVision software. Numbers of cells in each category are shown as a percentage of the total number of cells counted, and the SD is indicated.

Vacuolar pH Measurements

Vacuolar pH was measured using the ratiometric pH indicator dye BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) (Life Technologies) as described by Diakov et al. (2013). Data were collected using a FluoroLog-3 spectrofluorometer and FluorEssence software (HORIBA). See additional method details in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.10.014.

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REFERENCES

Baars, T.L., Petri, S., Peters, C., and Mayer, A. (2007). Role of the V-ATPase in regulation of the vacuolar fission-fusion equilibrium. Mol. Biol. Cell *18*, 3873–3882.

Baltscheffsky, M., Schultz, A., and Baltscheffsky, H. (1999). H+-proton-pumping inorganic pyrophosphatase: a tightly membrane-bound family. FEBS Lett. *452*, 121–127.

Bayer, M.J., Reese, C., Buhler, S., Peters, C., and Mayer, A. (2003). Vacuole membrane fusion: V0 functions after trans-SNARE pairing and is coupled to the Ca2+-releasing channel. J. Cell Biol. *162*, 211–222.

Conradt, B., Haas, A., and Wickner, W. (1994). Determination of four biochemically distinct, sequential stages during vacuole inheritance in vitro. J. Cell Biol. *126*, 99–110.

Cowles, C.R., Snyder, W.B., Burd, C.G., and Emr, S.D. (1997). Novel Golgi to vacuole delivery pathway in yeast: identification of a sorting determinant and required transport component. EMBO J. *16*, 2769–2782.

Darsow, T., Rieder, S.E., and Emr, S.D. (1997). A multispecificity syntaxin homologue, Vam3p, essential for autophagic and biosynthetic protein transport to the vacuole. J. Cell Biol. *138*, 517–529.

Diakov, T.T., and Kane, P.M. (2010). Regulation of vacuolar proton-translocating ATPase activity and assembly by extracellular pH. J. Biol. Chem. *285*, 23771–23778.

Diakov, T.T., Tarsio, M., and Kane, P.M. (2013). Measurement of vacuolar and cytosolic pH in vivo in yeast cell suspensions. J. Vis. Exp. (74)

Drake, R., Serrano, A., and Pérez-Castiñeira, J.R. (2010). N-terminal chimaeras with signal sequences enhance the functional expression and alter the subcellular localization of heterologous membrane-bound inorganic pyrophosphatases in yeast. Biochem. J. *426*, 147–157.

Finnigan, G.C., Cronan, G.E., Park, H.J., Srinivasan, S., Quiocho, F.A., and Stevens, T.H. (2012). Sorting of the yeast vacuolar-type, proton-translocating ATPase enzyme complex (V-ATPase): identification of a necessary and sufficient Golgi/endosomal retention signal in Stv1p. J. Biol. Chem. *287*, 19487–19500.

Graham, L.A., Flannery, A.R., and Stevens, T.H. (2003). Structure and assembly of the yeast V-ATPase. J. Bioenerg. Biomembr. *35*, 301–312.

Haas, A., Conradt, B., and Wickner, W. (1994). G-protein ligands inhibit in vitro reactions of vacuole inheritance. J. Cell Biol. *126*, 87–97.

Hiesinger, P.R., Fayyazuddin, A., Mehta, S.Q., Rosenmund, T., Schulze, K.L., Zhai, R.G., Verstreken, P., Cao, Y., Zhou, Y., Kunz, J., and Bellen, H.J. (2005). The v-ATPase V0 subunit a1 is required for a late step in synaptic vesicle exocytosis in *Drosophila*. Cell *121*, 607–620.

Kawasaki-Nishi, S., Nishi, T., and Forgac, M. (2001). Arg-735 of the 100-kDa subunit a of the yeast V-ATPase is essential for proton translocation. Proc. Natl. Acad. Sci. USA *98*, 12397–12402.

Martínez-Muñoz, G.A., and Kane, P. (2008). Vacuolar and plasma membrane proton pumps collaborate to achieve cytosolic pH homeostasis in yeast. J. Biol. Chem. *283*, 20309–20319.

Mozdy, A.D., McCaffery, J.M., and Shaw, J.M. (2000). Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. J. Cell Biol. *151*, 367–380.

Nichols, B.J., Ungermann, C., Pelham, H.R., Wickner, W.T., and Haas, A. (1997). Homotypic vacuolar fusion mediated by t- and v-SNAREs. Nature *387*, 199–202.

Perez-Castineira, J.R., Lopez-Marques, R.L., Villalba, J.M., Losada, M., and Serrano, A. (2002). Functional complementation of yeast cytosolic pyrophosphatase by bacterial and plant H+-translocating pyrophosphatases. Proc. Natl. Acad. Sci. USA *99*, 15914–15919.

Pérez-Castiñeira, J.R., Hernández, A., Drake, R., and Serrano, A. (2011). A plant proton-pumping inorganic pyrophosphatase functionally complements the vacuolar ATPase transport activity and confers bafilomycin resistance in yeast. Biochem. J. *437*, 269–278.

Peri, F., and Nüsslein-Volhard, C. (2008). Live imaging of neuronal degradation by microglia reveals a role for v0-ATPase a1 in phagosomal fusion in vivo. Cell *133*, 916–927.

Perzov, N., Padler-Karavani, V., Nelson, H., and Nelson, N. (2002). Characterization of yeast V-ATPase mutants lacking Vph1p or Stv1p and the effect on endocytosis. J. Exp. Biol. *205*, 1209–1219.

Peters, C., Bayer, M.J., Bühler, S., Andersen, J.S., Mann, M., and Mayer, A. (2001). Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion. Nature *409*, 581–588.

Piper, R.C., Bryant, N.J., and Stevens, T.H. (1997). The membrane protein alkaline phosphatase is delivered to the vacuole by a route that is distinct from the VPS-dependent pathway. J. Cell Biol. *138*, 531–545.

Plant, P.J., Manolson, M.F., Grinstein, S., and Demaurex, N. (1999). Alternative mechanisms of vacuolar acidification in H(+)-ATPase-deficient yeast. J. Biol. Chem. *274*, 37270–37279.

Sarafian, V., Kim, Y., Poole, R.J., and Rea, P.A. (1992). Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA *89*, 1775–1779.

Scott, C.C., Bissig, C., and Gruenberg, J. (2011). Duelling functions of the V-ATPase. EMBO J. *30*, 4113–4115.

Strasser, B., Iwaszkiewicz, J., Michielin, O., and Mayer, A. (2011). The V-ATPase proteolipid cylinder promotes the lipid-mixing stage of SNARE-dependent fusion of yeast vacuoles. EMBO J. *30*, 4126–4141.

Stroupe, C., Hickey, C.M., Mima, J., Burfeind, A.S., and Wickner, W. (2009). Minimal membrane docking requirements revealed by reconstitution of Rab GTPase-dependent membrane fusion from purified components. Proc. Natl. Acad. Sci. USA *106*, 17626–17633.

Takeda, K., Cabrera, M., Rohde, J., Bausch, D., Jensen, O.N., and Ungermann, C. (2008). The vacuolar V1/V0-ATPase is involved in the release of the HOPS subunit Vps41 from vacuoles, vacuole fragmentation and fusion. FEBS Lett. *582*, 1558–1563.

Tarsio, M., Zheng, H., Smardon, A.M., Martínez-Muñoz, G.A., and Kane, P.M. (2011). Consequences of loss of Vph1 protein-containing vacuolar ATPases (V-ATPases) for overall cellular pH homeostasis. J. Biol. Chem. *286*, 28089–28096.

Ungermann, C., Wickner, W., and Xu, Z. (1999). Vacuole acidification is required for trans-SNARE pairing, LMA1 release, and homotypic fusion. Proc. Natl. Acad. Sci. USA *96*, 11194–11199.

Wada, Y., Ohsumi, Y., and Anraku, Y. (1992). Genes for directing vacuolar morphogenesis in *Saccharomyces cerevisiae*. I. Isolation and characterization of two classes of vam mutants. J. Biol. Chem. *267*, 18665–18670.

Weisman, L.S., and Wickner, W. (1988). Intervacuole exchange in the yeast zygote: a new pathway in organelle communication. Science *241*, 589–591.

Wickner, W. (2010). Membrane fusion: five lipids, four SNAREs, three chaperones, two nucleotides, and a Rab, all dancing in a ring on yeast vacuoles. Annu. Rev. Cell Dev. Biol. *26*, 115–136.