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The V-ATPase proteolipid cylinder promotes the lipid-mixing stage of SNARE-dependent fusion of yeast vacuoles

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The V-ATPase V_0 sector associates with the peripheral V_1 sector to form a proton pump. V_0 alone has an additional function, facilitating membrane fusion in the endocytic and late exocytic pathways. V_0 contains a hexameric proteolipid cylinder, which might support fusion as proposed in proteinaceous pore models. To test this, we randomly mutagenized proteolipids. We recovered alleles that preserve proton translocation, normal SNARE activation and *trans*-SNARE pairing but that impair lipid and content mixing. Critical residues were found in all subunits of the proteolipid ring. They concentrate within the bilayer, close to the ring subunit interfaces. The fusion-impairing proteolipid substitutions stabilize the interaction of V_0 with V_1. Deletion of the vacuolar v-SNARE Nyv1 has the same effect, suggesting that both types of mutations similarly alter the conformation of V_0. Also covalent linkage of subunits in the proteolipid cylinder blocks vacuole fusion. We propose that a SNARE-dependent conformational change in V_0 proteolipids might stimulate fusion by creating a hydrophobic crevice that promotes lipid reorientation and formation of a lipidic fusion pore.

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Introduction

Membrane fusion is a key process in intracellular vesicular transport and organelle inheritance and homeostasis. It is catalysed by a conserved protein machinery. Yeast vacuoles are a useful model system to study membrane fusion that allows to dissect these reactions into individual phases and analyse the behaviour of the involved proteins in these intermediate states. Vacuole fusion begins with a priming step that comprises the ATP-dependent release of SNARE proteins from fusion-incompetent *cis*-SNARE complexes, driven by the AAA-ATPase Sec18/NSF. Priming releases the co-factor Sec17/α-SNAP from the membrane and facilitates the tethering of fusion partners supported by Ypt7p and the HOPS complex (Mayer and Wickner, 1997; Ungermann *et al*, 1998; Price *et al*, 2000; Hickey and Wickner, 2010). Tethering is necessary for subsequent tighter binding termed docking. During docking, t- and v-SNAREs from different vacuoles form *trans*-complexes that force the membranes into close apposition (Dietrich *et al*, 2003; Collins and Wickner, 2007; Schwartz and Merz, 2009). HOPS may stabilize these *trans*-SNARE complexes and enhance their fusogenic potential (Pieren *et al*, 2010; Xu *et al*, 2010). Transition from docked to fused vacuoles occurs via a hemifusion intermediate, which is characterized by lipid continuity between fusing organelles, the contents of which are still separated (Reese *et al*, 2005). Fusion can be reconstituted using vacuolar SNAREs alone but, depending on the conditions chosen, other factors such as HOPS or the Rab-GTPase Ypt7p can become necessary (Mima *et al*, 2008; Xu *et al*, 2010). Membrane fusion of intact vacuoles requires numerous further factors (Ostrowicz *et al*, 2008), among them the a subunit of the V_0 sector of the vacuolar H^+-ATPase (Peters *et al*, 2001; Bayer *et al*, 2003; Thorngren *et al*, 2004; Takeda *et al*, 2008).

The V-ATPase is a multiprotein complex catalysing ATP-driven proton transport from the cytosol into intracellular compartments or the extracellular space (Graham *et al*, 2003; Forgac, 2007; Li and Kane, 2009). It is composed of a peripheral sector (V_1) harbouring the ATPase activity and a membrane-integral sector (V_0), which translocates protons. The two sectors reversibly dissociate in a reaction regulated by glucose (Kane, 1995), the RAVE complex (Smardon *et al*, 2002) and microtubules (Xu and Forgac, 2001). Yeast V_0 contains six subunits, a (Vph1p/Stv1p), d (Vma6p), the proteolipids c (Vma3p), c′ (Vma11p) and c″ (Vma16p) and subunit e (Vma9p). All yeast V-ATPase subunits are encoded by a single gene, except subunit a. The a subunit isoform Vph1p is found on vacuoles whereas the other isoform, Stv1p, localizes mainly to the Golgi compartment (Graham *et al*, 2003). The proteolipids are highly conserved small transmembrane proteins spanning the membrane four times with both termini being in the vacuolar lumen (Powell *et al*, 2000). They form a hexameric ring with the stoichiometry 4 (Vma3p):1 (Vma11p):1 (Vma16p) with Vma11 and Vma16 at neighbouring positions (Wang *et al*, 2007). The denomination of these proteins as proteolipids is not due to a lipid modification but indicates that they are soluble in organic solvents, a property they share with lipids but which is quite unusual for proteins. V-ATPase-catalysed proton translocation occurs by rotation of the proteolipid ring along the a subunit, driven by ATP-dependent torque generated by V_1 (Graham *et al*, 2003; Forgac, 2007). Genetic or pharmacological disruption of V-ATPase function in yeast leads to the vma^- phenotype, which is characterized by severe growth

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defects and conditional lethality at alkaline pH and elevated Ca^{2+} concentrations, and by increased sensitivity to heavy metals and a multitude of small molecule compounds. The severe phenotypes resulting from V-ATPase pump deficiency restrict detailed analysis of V_0 function in fusion in yeast. In higher organisms, systemic V-ATPase disruption is even lethal. Only deletion of individual a subunit isoforms, whose expression is restricted to few tissues or cell types, can be tolerated (Sun-Wada *et al*, 2006; Marshansky and Futai, 2008; Williamson *et al*, 2010) and can then reveal specific functions of V-ATPase in these cell types. For this reason, analyses of V_0 function in fusion focused mainly on the a subunits so far.

V_0 was shown to promote fusion at multiple steps in the late exocytic and endocytic pathways in a variety of organisms, for example, fusion between vacuoles (lysosomes), secretion of neurotransmitters and insulin, phagosome–lysosome fusion and the secretion of exosomes (Peters *et al*, 2001; Bayer *et al*, 2003; Hiesinger *et al*, 2005; Liegeois *et al*, 2006; Sun-Wada *et al*, 2006; Dunant *et al*, 2009; Di Giovanni *et al*, 2010). V_0 interacts with SNAREs on synaptic vesicles in rat, fish and fly (Galli *et al*, 1996; Morel *et al*, 2003; Di Giovanni *et al*, 2010; Williamson *et al*, 2010) and on yeast vacuoles. During yeast vacuole fusion, V_0 sectors from the two fusion partners form stable associations (*trans*-complexes) (Peters *et al*, 2001; Müller *et al*, 2002; Bayer *et al*, 2003). A central element of V_0 is a pore-like proteolipid ring. Its structure resembles the arrangement of proteins postulated in pore models of membrane fusion (Lindau and Almers, 1995; Jahn and Grubmüller, 2002). This, together with the *trans*-complex formation of V_0 sectors during vacuole fusion, raised speculations that proteolipids might initiate a proteinaceous fusion pore that radially expands in a regulated manner (Peters *et al*, 2001). An alternative to the protein pore model is the stalk model, which suggests a small lipidic structure connecting fusing bilayers (Chernomordik *et al*, 2006). The mentioned fusion models represent extremes and hybrids between them can be envisioned (Jahn and Grubmüller, 2002; Jackson and Chapman, 2008).

In the work presented here, we undertook an extensive and unbiased mutational analysis of the V_0 proteolipids in vacuole fusion in order to overcome the limitations of the vma^- phenotype. We screened for point mutations that generate selective defects in vacuole fusion but support proton pump function and normal growth. We analysed the effects of these mutations on different molecularly defined stages of the fusion reaction such as SNARE activation (priming), *trans*-SNARE pairing (docking) and lipid and content mixing.

Results

Point mutations in proteolipids leading to vacuole fragmentation in vivo

We explored the function of proteolipids in vacuole fusion by random mutagenesis followed by a two-step screening procedure. The first step of screening demanded preservation of proton translocation activity. To this end, we plated the cells onto medium buffered to pH 7.5, which suppresses growth of clones lacking V-ATPase pump function (Graham *et al*, 2003). The second step visually screened fragmentation of vacuoles into small vesicles. This phenotype is frequently observed in mutants defective in vacuole fusion (Wada *et al*, 1992; Seeley *et al*, 2002) because a balance of fusion and fission activities determines size and copy number of vacuoles (LaGrassa and Ungermann, 2005; Weisman, 2006; Baars *et al*, 2007).

Randomly mutagenized libraries of 6000–30 000 alleles for each proteolipid were generated by error-prone PCR of the open reading frames of proteolipid subunits VMA3, VMA11 and VMA16. The libraries were inserted into shuttle expression vectors, amplified in *Escherichia coli* and transfected into yeast strains that had been deleted for the corresponding proteolipid gene. The conditions for error-prone PCR had been adjusted to produce variants with an average of 5–10 mutations/allele, resulting in 1–10 amino acid changes. Striking the right balance was critical because if the mutation rate were too low, the library would contain many alleles yielding wild-type morphology, which would enormously increase the effort for subsequent microscopic screening. Excessive mutation would mostly produce alleles already non-functional for V-ATPase activity and produce complex mutation patterns that are difficult to dissect. We calculated survival rates during the pH selection step as the ratio between colony numbers on pH 5.5, which permits growth of V-ATPase-deficient cells, and pH 7.5, which kills them. Survival reached 5–10% for VMA3 and 25–35% for VMA11 and VMA16, indicating that mutagenesis had been sufficiently harsh to inactivate pump function in a majority of the alleles. Surviving colonies on the pH 7.5 selection plates were picked, grown in 96-well plates and stained with the vital dye FM4-64 (Vida and Emr, 1995). The cells were transferred to new 96-well plates equipped with a glass bottom, enabling direct fluorescence microscopy of the living cells. Clones showing more numerous and smaller vacuoles than the respective wild-type controls were retained.

Despite their fusion defect, deletion mutants lacking entire V-ATPase subunits show a single enlarged vacuole (Graham *et al*, 2003). This is due to their vacuole fission defect, which results from disturbed proton translocation and prevails over the fusion defects (Baars *et al*, 2007). In our screen we circumvented this limitation by creating point mutants that preserve proton translocation activity and hence vacuolar fission. Knockouts of the V_0 alleles VMA3, VMA11 and VMA16 that had been rescued with a plasmid-borne wild-type allele of the deleted subunit (referred to as Rec3, Rec11 and Rec16) displayed one or two vacuoles in the majority (~80%) of the cells (Figure 1A). After mutagenesis, we found mutants with increased vacuole copy numbers for all three proteolipids (Figure 1B). Clones showing three or more vacuoles per cell at a frequency exceeding 50% were re-analysed, their plasmids were recovered and sequenced. In order to verify that the vacuolar fragmentation phenotype was linked to the mutagenized plasmid, we re-transformed the recovered plasmids into respective deletion mutants in three different strain backgrounds, BY4741, BJ3505 and DKY6281 and re-analysed them by microscopy. Only alleles reproducing the fragmented vacuole phenotype upon re-transformation were pursued further. We tested the efficiency of mutagenesis for each ORF by sequencing 10 randomly chosen mutagenized plasmids that had not undergone selection. The mutation patterns indicated that mutagenesis had equally affected all portions of the proteolipids (Figure 4D). Since most alleles selected in the screen contained multiple mutations (Supplementary Tables S1–S3) we dissected these mutations by individually introducing them into wild-type ORFs. The vacuolar phenotype of these single point mutants

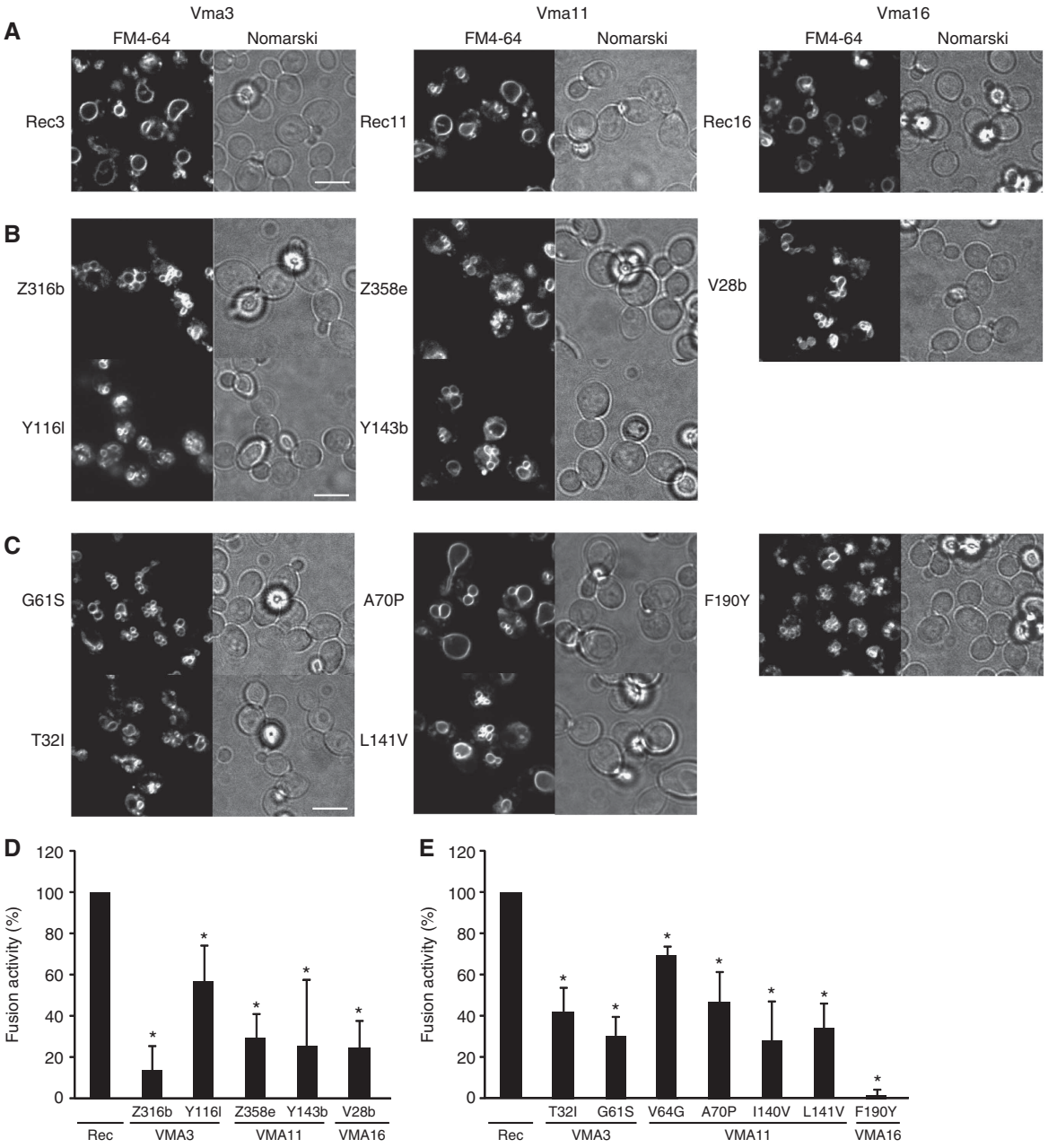


Figure 1 Vacuole structure and vacuole fusion activity in proteolipid mutants. BJ Δvma3, BJ Δvma11, BY Δvma16 reconstituted with plasmid-borne wild-type (designated as 'Rec x') (**A**) or mutant alleles (**B**, **C**) were grown in YPD pH 5.5 to logarithmic phase, stained with FM4-64 and analysed by confocal microscopy. Vacuoles were isolated from mutants found in the screen (**D**) and from strains expressing alleles with single substitutions derived from these mutants (**E**) and tested for their *in vitro* fusion activity relative to wild type. Vacuoles were incubated in standard fusion reactions in the presence of 1 mg/ml cytosol for 60 min on ice or at 27 °C. Then, ALP activities were determined as a tracer for fusion. ALP activity of the reconstituted wild types at 27 °C was 3.4–6.4 U for Vma3p, 2.4–5.7 U for Vma11p and 2.8–4.8 U for Vma16p. Ice values varied from 0.28–1.0 U for all experiments and were subtracted from respective 27 °C values ($n \geqslant 3$). *$P < 0.01$ for the difference of the marked value relative to the reconstituted wild type.

was analysed (Figure 1C). Single point mutations in VMA3 and VMA16 could be isolated that fully reproduced the fragmented vacuolar phenotype of the respective alleles with a complex mutation pattern. Single point mutants in VMA11 displayed a significant but weaker phenotype than the complex alleles Z358e and Y143b from which they had originated.

Vacuoles from proteolipid point mutants are fusion deficient

As *in vivo* experiments provide only qualitative and indirect information about the fusion activities of vacuoles, we analysed the mutants in an *in vitro* system allowing quantitation of fusion (Haas *et al*, 1994). Vacuoles were isolated from two yeast strains expressing either pro-alkaline phosphatase (pro-ALP; strains BJ3505 or BY4741) or the respective maturase (Pep4p, strain DKY6281). Upon fusion, the maturase cleaves and activates pro-ALP, the activity of which is then measured. We deleted proteolipids in these strain backgrounds and complemented them with the plasmids carrying mutant or wild-type alleles. We confirmed expression of pro-ALP by measuring ALP activity of BJ3505 vacuoles in the presence of 10 mM $ZnCl_2$, which bypasses the need for

proteolytic activation and renders pro-ALP active. For all tested point mutants in VMA3 and VMA11, the ALP reporter levels were comparable to wild type (Supplementary Figure S1). For the VMA16 mutant F190Y, they were reduced by 40%. Therefore, the content-mixing values were normalized to the pro-ALP levels in order to account for this effect. In order to exclude that Pep4p activity could be limiting, we incubated a mixture of BJ3505 and DKY6281 vacuoles in Triton X-100, which dilutes Pep4p, renders it limiting and allows its fusion-independent access to pro-ALP. pro-ALP maturation activity of all point mutants was at least as high as that of wild type (Supplementary Figure S1).

The *in vitro* fusion assays showed that the complex proteolipid mutant alleles recovered from the *in vivo* screen have significant fusion defects of 40–90% (Figure 1D). We also assayed the fusion activity of vacuoles from the single point mutants in the *in vitro* system. vma3^{T32I} and vma3^{G61S} showed 60–70% lower fusion activities than wild type (Figure 1E). Resolution of the substitutions in one of the screened mutants, vma3$^{T32S,\ I110V}$ (Y97j) showed that an individual vma3^{I110V} mutation affected vacuole fusion only weakly and that vma3^{T32S} alone had a moderate effect (not shown). We noted, however, that a vma3^{T32I} mutation, which is known to be functional for proton translocation (Bowman *et al*, 2004, 2006), by itself had a strong effect, confirming the relevance of the identified residue T32. Therefore, we used vma3^{T32I} for further functional analysis of this site. The mutant Y116l had lost 43% of wild-type fusion activity (Figure 1D). This mutant contains V44M, G79D and T86A substitutions that could not be resolved. Individual G79D or T86A substitutions did not impede fusion *in vitro* and did not alter vacuole morphology *in vivo*. The V44M substitution, on the other hand, inhibited fusion but induced the vma$^-$ phenotype. This suggests that the vma$^-$ phenotype of V44M is suppressed by the other two mutations and that vacuolar fragmentation arises from a cooperative effect of the three substitutions. By separating the mutations of vma11 mutants Z358e and Y143b, we found four substitutions to produce fusion defects. vma11^{V64G}, vma11^{A70P}, vma11^{I140V} and vma11^{L147V} decreased fusion activity by 31, 53, 72 and 67%, respectively. The substitution vma16^{F190Y} proved to be causative for the fusion defect in mutant V28b. vma16^{F190Y} reduced fusion activity by >90% relative to wild type. We blotted purified vacuoles from the mutants against the mutagenized V-ATPase subunits (Supplementary Figure S2) and against a great variety of other vacuolar fusion factors, such as the SNAREs Nyv1p, Vam3p, Vam7p and Vti1p, the NSF-homologue Sec18p and its co-factor Sec17/α-SNAP, the HOPS complex and the Rab-GTPase Ypt7p (Supplementary Figure S2). The levels of these proteins in the mutant vacuoles were similar to those found in wild type. These data hence suggest a direct role of proteolipids in vacuole fusion and identify a spectrum of residues in the V_0 proteolipids that are critical for this reaction.

Fusion-deficient proteolipid substitutions support vacuolar acidification

We tested V-ATPase proton pump activity in strains carrying the fusion-deficient proteolipids with single point mutations that had been identified above. Since yeast cells require V-ATPase pump activity for growth on slightly alkaline media but not for growth on acidic media, we spotted dilution series of the cells onto YPD plates buffered to pH 7.5 or pH 5.5 (Figure 2). The isolated point mutants grew like wild type in both conditions whereas the strains deleted for the respective proteolipids failed to grow at pH 7.5. We also measured V-ATPase-dependent proton translocation activity directly, using isolated vacuoles and the acidophilic dye 9-amino-6-chloro-2-methoxyacridine (ACMA) (Casadio, 1991). ACMA concentrates in vacuoles as a function of the vacuolar proton gradient, leading to fluorescence self-quenching. Upon addition of ATP, fluorescence of a suspension of wild-type vacuoles decreased by 70% (Figure 3). Fluorescence self-quenching was suppressed by co-incubation with the V-ATPase inhibitor concanamycin A and the quenched state could be reverted by adding the protonophore carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) at the end of the incubation. This validates the assay. In line with the *in vivo* observations, all proteolipid substitutions produced vacuoles showing significant proton translocation activity *in vitro*. vma11^{A70}, vma11^{L141V} and vma16^{F190Y} signals were 30–40% lower than that of the wild type (Figure 3C and D). We consider this reduction as not relevant since 60–70% of wild-type pump activity support normal growth. Furthermore, even complete inhibition of V-ATPase pump activity by concanamycin A does not reduce fusion activity of the vacuoles (Supplementary Figure S3). This suggests that proteolipid alleles supporting a robust proton pump function can cause fusion defects *in vitro* and fragmented vacuoles *in vivo*. They genetically separate functions of the proteolipid cylinder in ATP-dependent proton translocation and vacuole fusion.

Fusion-relevant residues cluster in the transmembrane domains at proteolipid interfaces

We constructed a homology model of the proteolipid cylinder from yeast based on the X-ray structure of the V-ATPase proteolipid ring from *Enterococcus hirae* (PDB 2bl2) and the proteolipid arrangement proposed by a study of yeast proteolipids (Murata *et al*, 2005; Wang *et al*, 2007). The model does not contain transmembrane domain 1 of Vma16p as no homologous region exists in *E. hirae*. This domain is not required for the proton translocation function of the V-ATPase (Nishi *et al*, 2003) and we observed that it is also dispensable for vacuole fusion (not shown). In the structural model, the residues affected by candidate mutations (shown as yellow spheres) cluster close to the centre of the transmembrane domains (Figure 4A and B). Using the structural information of the homology model, we constructed a helical wheel projection of the proteolipid cylinder (Figure 4C). Six of the seven identified candidate mutations protrude into the space between the proteolipids. They might affect the interactions between the subunits. In order to exclude a potential bias towards that region in the mutagenesis, we sequenced 10 randomly selected clones from each proteolipid library that had not undergone the selection procedure (Figure 4D). In these non-screened clones, the mutations are evenly distributed throughout all domains of the protein. This suggests that the clustering of the selected residues is a specific consequence of our screening for vacuolar fusion defects, highlighting a region of the proteolipids that is critical for this function. Most of the affected residues are conserved in proteolipid homologues from higher eukaryotic organisms (Supplementary Figure S4).

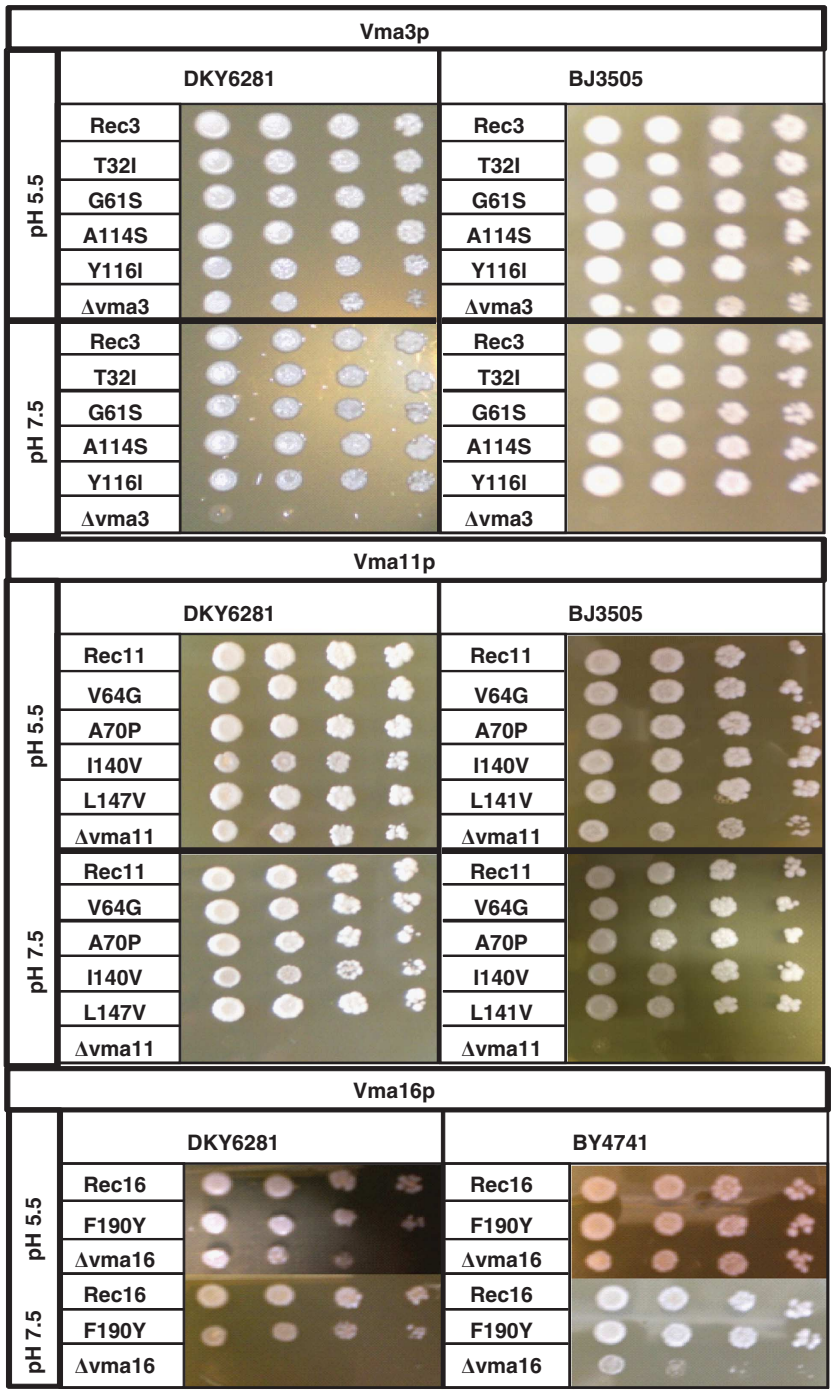


Figure 2 Fusion-deficient proteolipid alleles rescue yeast growth at alkaline pH. Wild-type and mutant strains were grown to logarithmic phase in selective medium. Cultures were diluted to an OD_{600} of 0.2. Drops of 1.5 µl of 1:7 serial dilutions were spotted on YPD plates buffered to pH 5.5 (50 mM MES) or pH 7.5 (50 mM HEPES). Pictures were taken after 48 h of incubation at 30 °C.

Proteolipid mutations interfere with the fusion pathway prior to lipid flow

Vacuole fusion occurs in a series of subreactions resulting in *cis*-SNARE complex disruption (priming), *trans*-SNARE complex formation (docking) and fusion. Fusion proceeds through a hemifusion intermediate, which is defined as a state in which the outer leaflets merge but the inner leaflets still separate the compartments and prevent content mixing (Reese *et al*, 2005). We tested whether V_0 proteolipid mutations affected hemifusion or a preceding step. To this end, a lipid-mixing assay based on rhodamine dequenching was combined with the ALP-based content-mixing assay. Self-quenching concentrations of rhodamine–phosphatidylethanolamine (Rh–PE) were incorporated into isolated vacuoles. Labelled vacuoles were mixed with an excess of unlabelled vacuoles and reactions were split in order to analyse content mixing and lipid mixing in parallel. Lipid mixing was measured via the increase in specific fluorescence that results

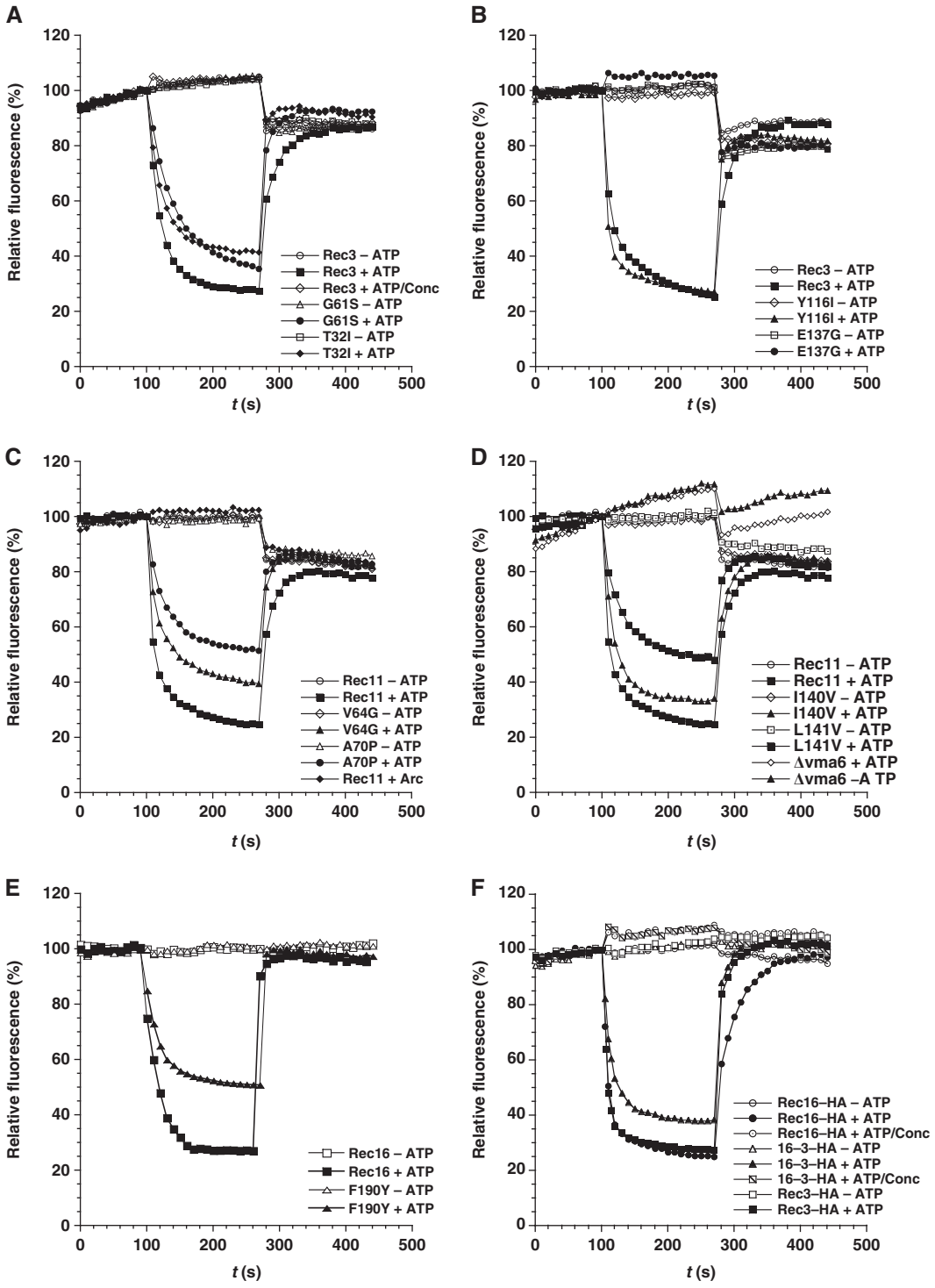
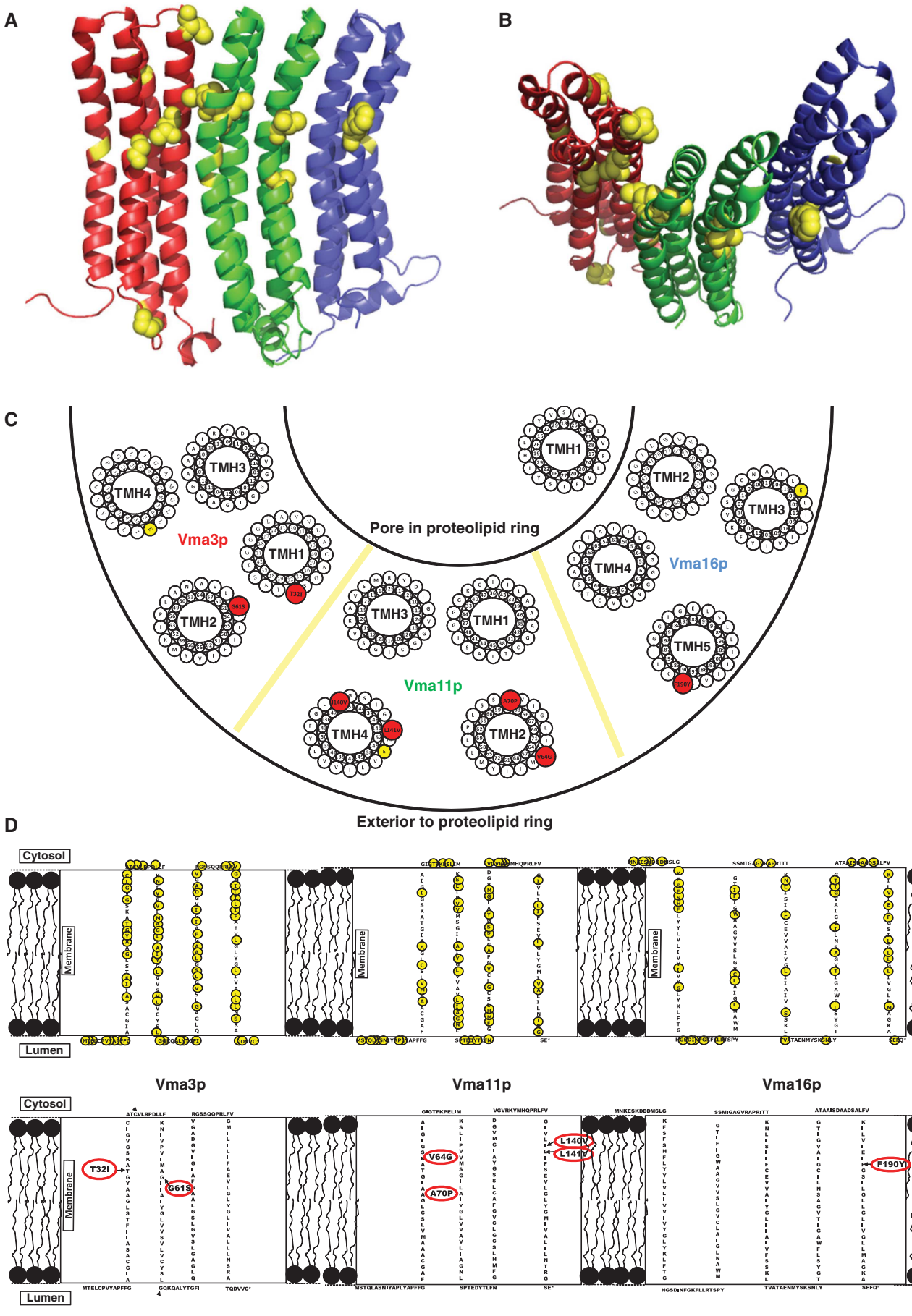


Figure 3 Fusion-deficient proteolipid alleles support proton translocation in isolated vacuoles. Vacuoles were prepared from cells expressing proteolipids with the indicated substitutions in Vma3p (**A**, **B**), Vma11p (**C**, **D**) or Vma16p (**E**), a fusion protein of Vma16p and Vma3p (16–3–HA), or proteolipids carrying an HA-tag (Rec3–HA, Rec16–HA) (**F**). In each case, the expressed allele was the sole source of the respective proteins in the cell. The vacuoles were used to measure H^+ translocation activity via fluorescence quenching of ACMA. Vacuoles were incubated with ACMA for 2 min in fusion buffer without ATP. Then, ATP was added and fluorescence was measured for 3 min, followed by addition of FCCP and recording for further 3 min. Control reactions were run in the absence of ATP, in the presence of 0.4 μM concanamycin A (Conc) or 12 μM archazolide (Arc), or using vacuoles from the subunit d deletion mutant Δvma6 or from $vma3^{E137G}$, a strain lacking the H^+-carrying glutamate.

from Rh–PE dilution, caused by fusion of labelled with unlabelled vacuoles. The fluorescence increase was normalized to the fluorescence after maximal dequenching, which was obtained by solubilizing the samples with 0.5% Triton X-100 (Reese *et al*, 2005). The proteolipid point mutants showed clearly lower dequenching than corresponding wild-type curves (Figure 5A–E). Lipid mixing and content mixing were reduced to similar degrees in the mutants



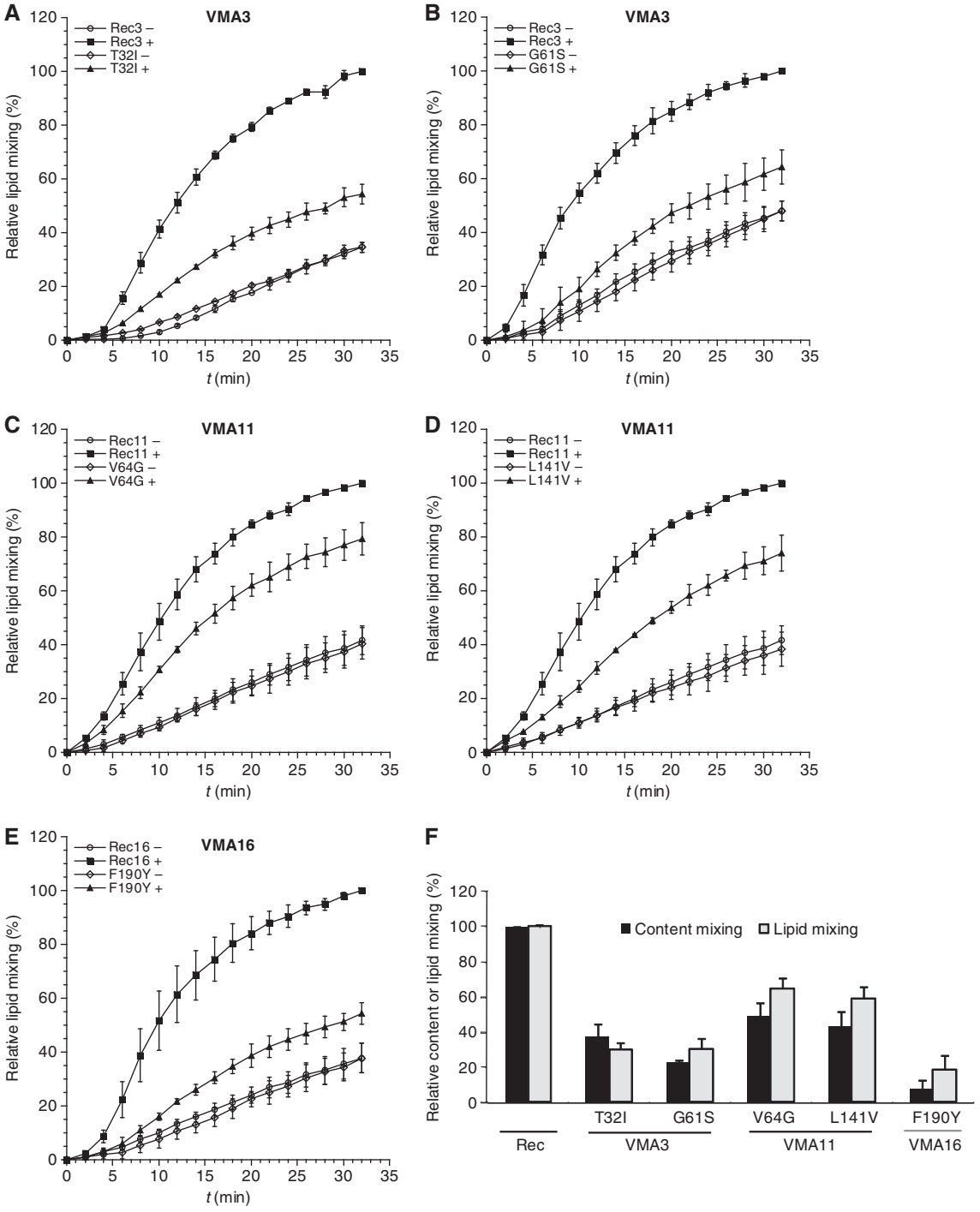


Figure 5 Proteolipid substitutions impede lipid flow. Vacuoles were labelled with Rh–PE at self-quenching concentrations, mixed with a six-fold excess of unlabelled vacuoles and incubated under fusion conditions in the presence or absence of ATP. Fluorescence was monitored for mutants in VMA3 (**A**, **B**), VMA11 (**C**, **D**) and VMA16 (**E**). (**F**) At the end of the fusion reactions, content-mixing signals were determined for all samples using the ALP assay. Lipid-mixing activities were calculated from the initial slope of the dequenching curves form (**A**–**E**). Then, content-mixing and lipid-mixing activities were normalized to those of the wild-type cells in order to facilitate comparison ($n = 3$).

Figure 4 Positioning of substitutions affecting vacuole fusion. Side view (**A**) and top view (**B**) of a homology model of the proteolipid cylinder, based on the proteolipid structure 2bl2 from *E. hirae* (Murata *et al*, 2005). Only half of the cylinder is shown for clarity. The positions in the cylinder that are not shown are occupied by three further copies of the c-subunit Vma3p. Mutations are marked as yellow spheres in the ribbon representations of Vma3p (red), Vma11p (green) and Vma16p (blue). The essential glutamic acid residues are shown as yellow patches within the ribbons. (**C**) Helical wheel projections of the proteolipid transmembrane domains. The orientation of the helical wheels corresponds to that of the transmembrane domains in the top view of the homology model. Residues affecting fusion are coloured in red. (**D**) Sequence coverage of the mutagenesis approach. Plasmids were retrieved from 10 random clones of each mutagenized proteolipid library that had not undergone the screening procedure. The ensemble of affected residues found in all 10 clones is labelled as yellow spheres in each of the proteolipid sequences (upper panel). Their random distribution contrasts the clustering of fusion-deficient point mutations in the cytosolic half of the transmembrane domains, shown for comparison (lower panel).

(Figure 5F), suggesting that none of the mutations affected the reaction between lipid transition and content mixing. Blocks at this stage are indicative of a problem in fusion pore opening and can be detected by comparison of these two assays (Reese and Mayer, 2005; Reese *et al*, 2005; Pieren *et al*, 2010). The proteolipids should hence have a role that can precede lipid mixing or coincide with it.

Proteolipid mutants can maintain normal priming and trans-SNARE pairing

SNAREs residing within one vacuole form *cis*-complexes. These *cis*-SNARE complexes are disrupted by the ATP-dependent chaperone Sec18/NSF and its co-chaperone Sec17p/α-SNAP (Mayer *et al*, 1996; Ungermann *et al*, 1998). Sec17p is released during this activation reaction. We assayed the ATP-dependent release of Sec17p as a reporter for priming. Fusion reactions were started by addition of ATP and, after 10 min, the vacuoles were sedimented and the pellets and supernatants blotted for Sec17p. The wild type and all tested proteolipid point mutants showed similar behaviour, that is, the majority of vacuole-bound Sec17p was released into the supernatant within 10 min after ATP addition (Figure 6A). In the absence of ATP, Sec17p was not released. Therefore, all mutant vacuoles prime *cis*-SNARE complexes normally.

The subsequent docking step was measured via *trans*-SNARE complex formation. To this end, equal amounts of two differently tagged vacuole populations were mixed. One contained the v-SNARE Nyv1p with an HA-tag, the other one the t-SNARE Vam3p with a VSV-tag. After 30 min of incubation under fusion conditions, we solubilized the membranes and precipitated Nyv1–HA. Analysis of the co-precipitated t-SNARE allows to distinguish between SNARE complexes *in cis* (Nyv1p–HA/Vam3p) and *in trans* (Nyv1p–HA/Vam3p–VSV) (Pieren *et al*, 2010) (Figure 6B). We could not obtain a $Vma16^{F190Y}$ mutant expressing Nyv1–HA at normal level on the vacuole. We could, however, obtain tagged SNAREs at normal vacuolar expression levels for the other substitutions, which were then analysed for *trans*-SNARE pairing. $vma3^{T32I}$ and $vma3^{G61S}$ vacuoles formed *trans*-SNARE complexes with similar efficiency as wild type (Figure 6C). $vma11^{V64G}$ also displayed similar efficiency as wild type, whereas $vma11^{L141V}$ decreased *trans*-SNARE pairing by 50%. Only weak *trans*-SNARE signals were detected in reactions in which the vacuolar Rab-GTPase Ypt7p was inhibited with 0.1 mg/ml GTP dissociation inhibitor (Gdi1p) or which had been incu-

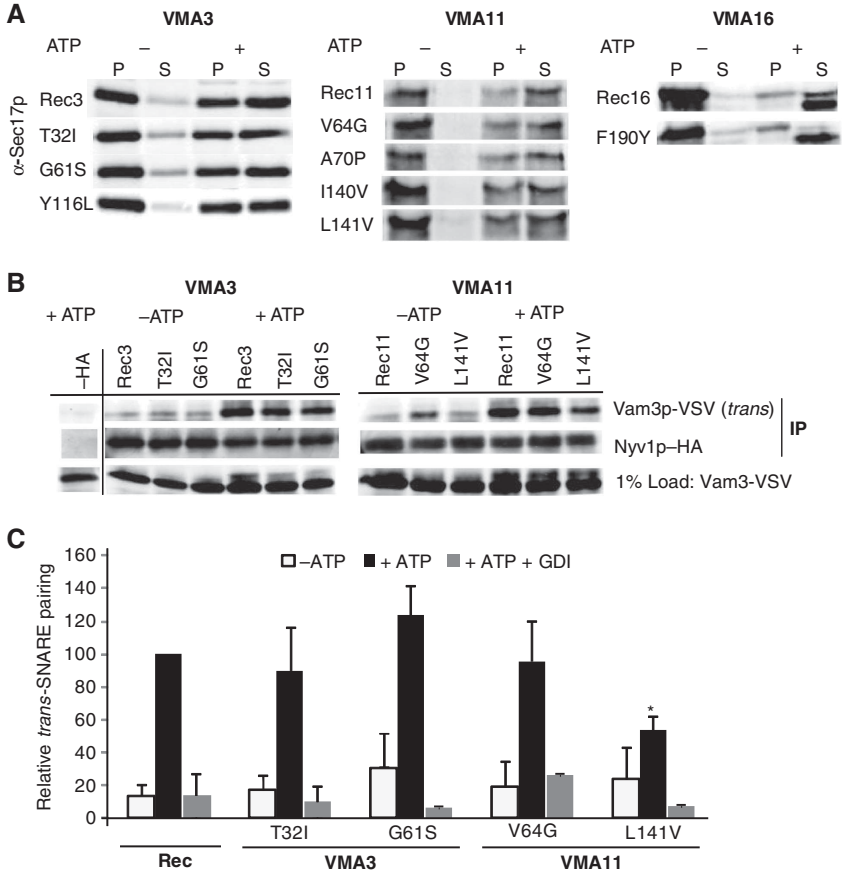


Figure 6 Activation and *trans*-complex formation of SNAREs. (**A**) SNARE activation. Fusion reactions with vacuoles from proteolipid point mutants were incubated in the presence or absence of ATP (15 min, 27 °C) and centrifuged. Supernatants and pellets were separated, TCA-precipitated and analysed for the presence of Sec17p/α-SNAP by western blot. Vacuoles from all analysed candidate mutants released Sec17p/α-SNAP in presence of ATP. (**B**) *Trans*-SNARE pairing. Vacuoles were prepared from proteolipid mutants carrying either Nyv1–HA or Vam3–VSV. The two vacuole populations were mixed into one fusion reaction that was incubated in the presence or absence of ATP (30 min, 27 °C). After solubilization in Triton X-100, Nyv1–HA was adsorbed to anti-HA beads. Adsorbed proteins were analysed by SDS–PAGE and western blotting. *Trans*-SNARE pairing is assessed via the amount of Vam3–VSV from one fusion partner that co-adsorbs with Nyv1–HA from the other fusion partner. (**C**) The signals from (**B**) were detected by fluorescent secondary antibodies and quantified with a Licor Odyssey scanner ($n=3$). $^*P<0.01$ for the difference of the marked value relative to the reconstituted wild type.

bated in the absence of ATP. These data suggest that vma11^{L141V} inhibits fusion at the stage of *trans*-SNARE pairing whereas the vma3^{T32I}, vma3^{G61S} and vma11^{V64G} substitutions inhibit a stage subsequent to or independent of *trans*-SNARE pairing.

Proteolipid point mutations change the V_0 conformation

V-ATPases can dissociate into free V_0 and V_1 sectors in a controlled fashion (Kane, 1995). We reasoned that if proteolipid mutations influenced the conformation of V_0, this might lead to detectable changes in V_0/V_1 association. Since V_1 sectors bind to vacuoles only by assembly onto a V_0 sector (Graham *et al*, 2003), the quantity of V_1 present on purified vacuoles can serve as a readout for V-ATPase assembly. Western blot analysis demonstrated that vacuoles from vma16^{F190Y} and vma3^{G61S} carry 4–5 times higher amounts of the V_1 subunit Vma1p than wild-type vacuoles (Figure 7A and B). By contrast, the amounts of the V_0 subunits Vph1p and Vma6p are similar and the total cellular levels of Vma1p remained unchanged in the mutants. This suggests a stabilization of the holoenzyme on vma16^{F190Y} and vma3^{G61S} vacuoles. Since SNAREs interact with V_0 (Galli *et al*, 1996; Peters *et al*, 2001; Morel *et al*, 2003; Hiesinger *et al*, 2005;

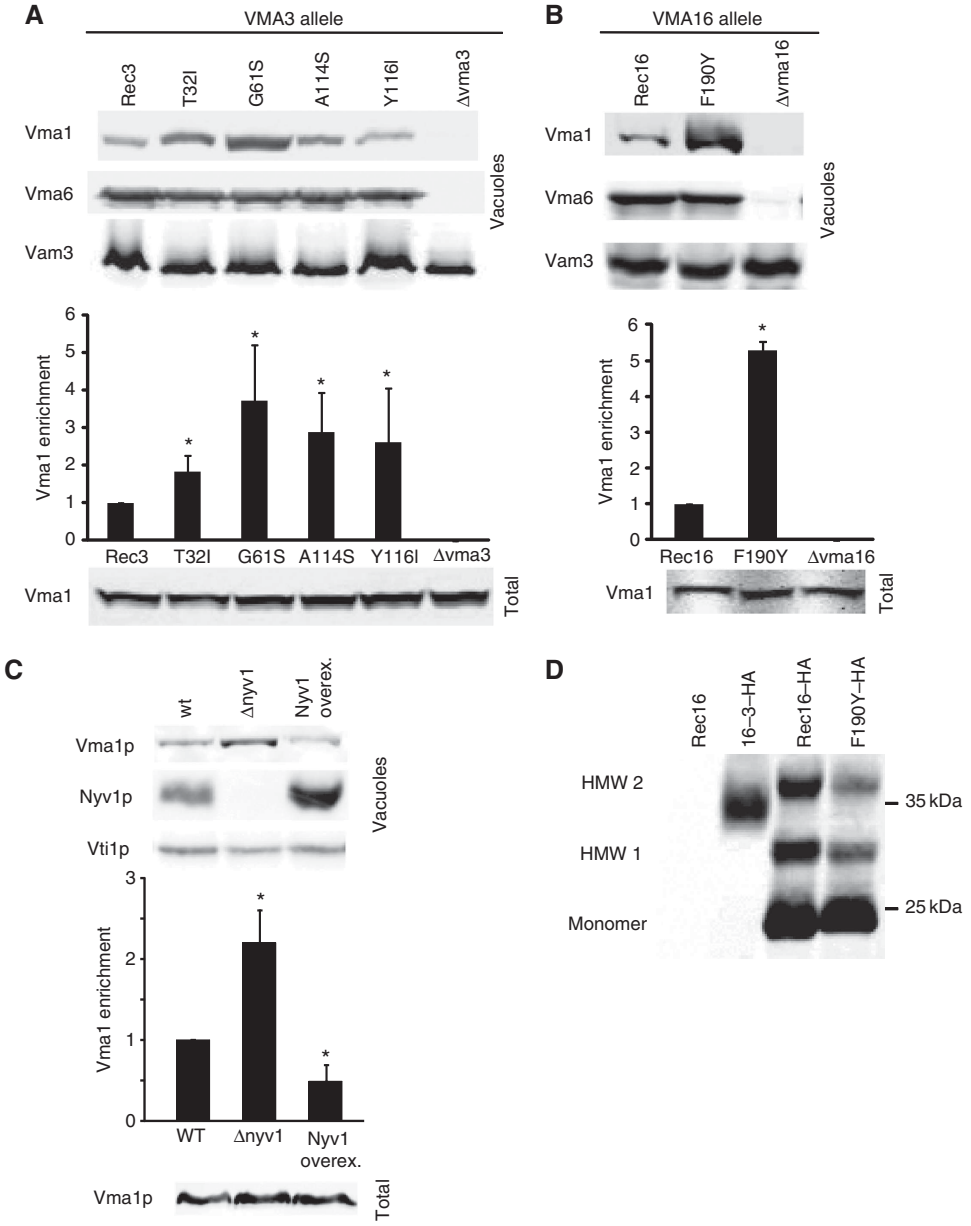


Figure 7 Vma1p levels on vacuoles are increased by proteolipid mutations and by deletion of NYV1. Vacuoles were prepared from the indicated mutant cells for (**A**) VMA3, (**B**) VMA16 and (**C**) or from cells lacking NYV1 (Δnyv1) or overexpressing NYV1 from the ADH promotor (NYV1 overex.), and from their respective wild-type equivalents. Spheroplast samples were taken during vacuole isolation to control protein levels in entire cells (total). Proteins were analysed by SDS–PAGE and western blot. The V_0 subunit Vma6p and the SNAREs Vam3p or Vti1p were used as loading controls. *$P<0.01$ for the difference of the marked value relative to the reconstituted wild type. $n=>3$. (**D**) HA-tagged VMA16 with or without the substitution F190Y or genetically fused to VMA3 were expressed in BJ3505. As a control for specificity of the antibodies, an untagged VMA16 was expressed in BJ3505. Vacuoles were isolated from these strains and the Vma16p variants were precipitated with antibodies to the HA epitope. Immunoprecipitates were analysed by western blotting for HA-tagged Vma16p variants.

Takeda *et al*, 2008; Di Giovanni *et al*, 2010), we also tested whether vacuolar SNAREs influenced V_1 attachment. Intriguingly, deleting the gene for the v-SNARE Nyv1p led to a significant enrichment of Vma1p on isolated vacuoles whereas the amount of V_0 remained unchanged (Figure 7C). Conversely, overexpression of NYV1 reduced the amount of Vma1p bound to vacuoles. This demonstrates a crosstalk between the V-ATPase and this SNARE and suggests that Nyv1p destabilizes the interaction of V_1 and V_0.

The proteolipids form oligomers that can resist dissociation by SDS (Umemoto *et al*, 1991; Hirata *et al*, 1997). We could detect such oligomers for HA-tagged variants of Vma16p by western blotting (Figure 7D). Vacuoles carrying the wild type (Rec16–HA) showed the monomeric protein and two additional bands at a higher molecular weight. These bands were absent in strains expressing a non-tagged Vma16p or a VMA16–VMA3 fusion protein (16–3–HA), excluding that they arose from crossreacting material unrelated to Vma16p. In $vma16^{F190Y}$, which affects the Vma16p–Vma11p interface, the higher molecular weight forms were 50 % less abundant whereas monomeric Vma16p appeared as in wild type. This suggests that $vma16^{F190Y}$ influences the interaction of the proteolipid subunits.

Pore models of membrane fusion had inspired speculations that an activity of the proteolipid cylinder in vacuole fusion might involve conformational changes and partial dissociation of the subunits in order to create clefts between them that could change the orientation of lipids and stimulate fusion (Lindau and Almers, 1995; Peters *et al*, 2001). In order to test the potential relevance of a dissociation or a significant conformational change of the cylinder, we could exploit earlier observations that Vma16p and Vma3p can be covalently linked by gene fusion (Flannery *et al*, 2004; Wang *et al*, 2007). We expressed a VMA16–VMA3 fusion ORF, tagged with an HA sequence (Flannery *et al*, 2004), in Δvma16 strains. Vacuoles from this strain had a strong fusion defect *in vitro* (Figure 8B). *In vivo*, the mutants showed a fragmented vacuolar phenotype (Figure 8A). The HA-tag itself does not inhibit vacuole fusion because monomeric Vma16p–HA supported vacuole fusion as well as the non-tagged Vma16p (Figure 8B). Furthermore, the VMA16–VMA3 fusion protein supports proton pump function (Figure 3F) (Flannery *et al*, 2004). This suggests that manipulating subunit contacts in the proteolipid cylinder by point mutations or by covalent linkage leads to fusion defects.

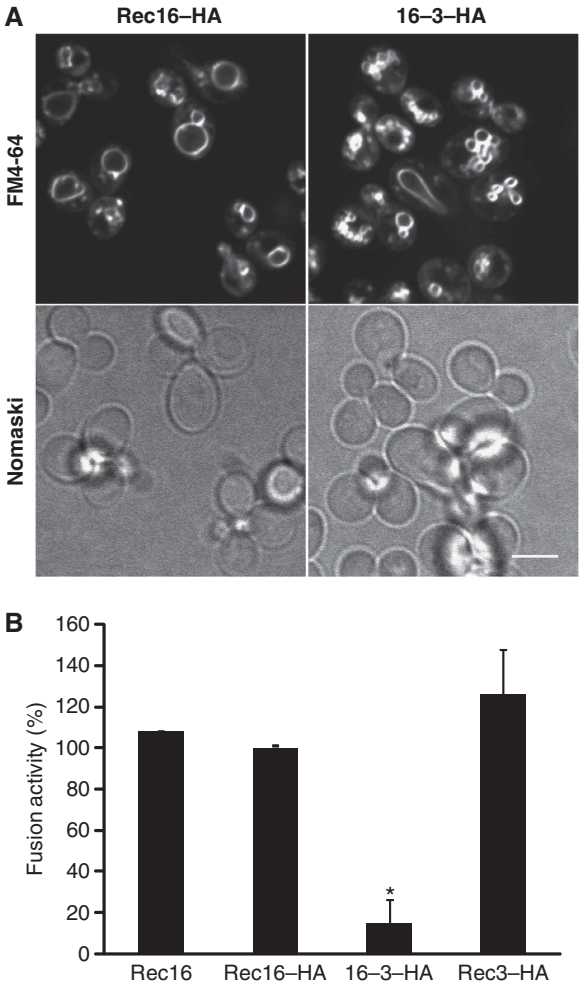


Figure 8 Fusion defect of a covalently linked Vma16–Vma3 dimer. (**A**) BY4741 Δvma16 cells were reconstituted with HA-tagged alleles of VMA16 (Rec16–HA) or of a gene fusion of VMA16 and VMA3 (16–3–HA). Cells were grown in YPD pH 5.5 to logarithmic phase, stained with FM4-64 and analysed by spinning disc confocal microscopy. (**B**) Vacuoles were isolated from DKY6281 and BY4741Δpep4 expressing VMA16 (Rec16), VMA16–HA or the VMA16–VMA3 gene fusion as unique source of Vma16p and from BJ3505 and DKY6281 expressing VMA3–HA as unique source of Vma3p (Rec3-HA). Fusion activities were determined as in Figure 2 and are shown relative to Vma16–HA. ALP activity of this reference at 27 °C was between 7.5–9.5 U. Ice values varied from 0.25 to 1.8 U for all experiments and were subtracted from respective 27 °C values. $^*P<0.01$ for the difference of the marked value relative to the reconstituted wild type ($n=3$).

Discussion

V_0 a subunits affect fusion reactions at multiple stations of the late exocytic and endocytic pathways (Peters *et al*, 2001; Hiesinger *et al*, 2005; Liegeois *et al*, 2006; Sun-Wada *et al*, 2006; Peri and Nusslein-Volhard, 2008; Di Giovanni *et al*, 2010). Investigation of the role of V_0 in fusion has remained mostly restricted to the a subunit because in many organisms, systemic loss of V-ATPase is lethal but the a subunits often exist in multiple isoforms. Thus, viable isoform deletion mutants can be obtained that affect only a certain tissue. It has not been clear whether the entire V_0 sector is necessary for fusion or whether this is a specific function of the a subunits. With knockout mutants, this distinction is difficult to make because deletion of one V_0 subunit often severely reduces the abundance of the other V_0 subunits (Graham *et al*, 2003). In order to obtain specific point mutations that preserve V_0 integrity, we screened for proteolipid alleles that interfere with fusion but support proton translocation. This also avoids side effects that might arise from the loss of V-ATPase pump activity. Since the identified residues are conserved from yeast to man, they might be transferable and allow analyses of proteolipid function also in multicellular organisms. A constraint is that several of the individual point mutations produce significant reductions in fusion activity but not as severe reductions as deletion mutants. This may be an inevitable consequence of our demand for structural integrity and, as we hypothesize below, the fact that the entire proteolipid ring rather than one defined subunit participates in fusion.

Several roles of the proteolipids in vacuole fusion can be envisioned, which are not mutually exclusive. They might act

indirectly by their function in proton pumping, they might promote localization, activation, or *trans*-complex formation of SNAREs, or they might participate in membrane merger itself. Indirect action via proton translocation is ruled out by the genetic dissociation of the pump and fusion activities and by the fact that pump activity can be pharmacologically suppressed without inhibiting vacuole fusion. It is conceivable that the hexameric proteolipid cylinder, which can interact with SNAREs (Galli *et al*, 1996; Morel *et al*, 2003; Di Giovanni *et al*, 2010), might offer several similar binding sites for SNAREs and locally concentrate them to enhance their fusogenic potential. Such a scenario is difficult to reconcile with the fact that a mutation in a single subunit of the hexameric cylinder can have profound effects on fusion. Single substitutions in Vma11p and Vma16p, which exist only in one copy per hexameric cylinder, should affect only one out of six potential binding sites, yet they can substantially impair fusion and have similar effects as substitutions in Vma3p, which exists in four copies. Furthermore, we found no significant changes in the interactions of proteolipids and SNAREs in immunoprecipitations from the mutant vacuoles. Thus, it appears unlikely that the proteolipids could act solely by localizing or concentrating SNAREs.

Our observations support separable roles of the proteolipids in SNARE pairing and in lipid mixing. Several mutants showed reduced fusion activity but normal SNARE activation and *trans*-SNARE pairing and thus genetically dissociate these events. This underlines the function of proteolipids in the lipid-mixing stage, which follows *trans*-SNARE pairing. That proteolipids can nevertheless influence SNARE pairing is suggested by vma11^{L141V}, which reduces the abundance of *trans*-SNARE complexes by half (Figure 6E). This reduction might reflect a lower efficiency of SNARE complex formation or reduced stability of SNARE complexes. It might originate from incomplete SNARE zippering, which may influence fusion (Chen *et al*, 1999; Melia *et al*, 2002; Zhang *et al*, 2005; Rizo *et al*, 2006; Sorensen *et al*, 2006; Schwartz and Merz, 2009), or from weakened interactions with the HOPS complex and its SM protein Vps33. She SM protein Vps33p promotes both SNARE complex assembly (Sato *et al*, 2000; Collins *et al*, 2005) and the terminal step of vacuole fusion, pore opening (Pieren *et al*, 2010). It was proposed that the interaction with the large SM protein containing HOPS complex might force SNARE complexes into a twisted orientation that would enable them to exert strain on their transmembrane domains, perturbing lipid structure and promoting fusion pore opening. Such a mechanism would convert the size of the complex into an advantage rather than an obstacle to fusion (Pieren *et al*, 2010). Neither partial zipping of SNAREs nor SNARE complex twisting can be measured in physiological membranes at present, so these possibilities could not be experimentally addressed in our study. Since vma11^{L141V} is buried within the bilayer, this substitution could not influence SNARE zipping or twisting by a direct interaction with the hydrophilic SNARE domains. A more plausible scenario is a conformational effect of vma11^{L141V} that could be transmitted to other V_0 subunits, which interact with SNAREs via their hydrophilic domains. A prime candidate would be the a subunit, which is in direct contact with the proteolipids, and offers a large hydrophilic domain, which interacts with t-SNAREs (Peters *et al*, 2001; Hiesinger *et al*, 2005; Williamson *et al*, 2010).

We also found indications for an influence in the other direction, from SNAREs on V_0. Deletion of the vacuolar v-SNARE gene NYV1 increased the binding of V_1 to vacuoles, similarly as the proteolipid point mutants did, and NYV1 overexpression reduced V_1 binding. This suggests that both the proteolipid point mutations and absence of Nyv1p leave V_0 in a similar conformation that does not support fusion and stabilizes the V_0/V_1 interaction. In agreement with our results, also the exocytic v-SNARE synaptobrevin binds proteolipids and interference with this interaction by competing peptides influences neurotransmitter release (Galli *et al*, 1996; Morel *et al*, 2003; Di Giovanni *et al*, 2010). This opens the interesting possibility that SNAREs not only passively bind to V_0 sectors but that they might actively transform the V-ATPase, either by competing with the attachment of V_1 to V_0 or by changing V_0 conformation to weaken its interaction with V_1. A detailed analysis of this aspect is under way.

Since several proteolipid mutants stabilize the V_0/V_1 holoenzyme, their fusion deficiency could result from a depletion of the pool of free V_0. The holoenzyme is a bulky structure of >800 kDa and due to its size it might interfere with the recruitment of V_0 to the fusion site. Several facts argue against this possibility: Isolated vacuoles contain $<20\%$ of V_0 in the form of holoenzymes; V_0 is over 10 times more abundant than the vacuolar t-SNAREs; the expression of vacuolar proteolipids must be reduced by at least 95% in order to block fusion; and about half of the holoenzymes on isolated vacuoles dissociate during the first 15 min of the fusion reaction, even for vacuoles of the proteolipid point mutants. Thus, it is unlikely that the supply of free V_0 limits fusion of the proteolipid mutants.

An alternative hypothesis that could correlate increased holoenzyme abundance with fusion defects is that V_0/V_1 holoenzymes might bind SNAREs and impair full zippering of *trans*-SNARE complexes by steric hindrance. The dimensions of a V_0/V_1 holoenzyme argue against this notion. V_0/V_1 protrudes >15 nm from the membrane (Zhang *et al*, 2008), a distance that could not be spanned by two interacting SNARE domains. Therefore, if the sequestration of SNAREs by V_0/V_1 holoenzymes caused the fusion defect by steric hindrance, the proteolipid mutations should have prevented or significantly reduced *trans*-SNARE pairing. This was not observed for most of them.

Electron microscopic studies revealed two conformations of the V_0 proteolipid cylinder, leading to the suggestion of considerable flexibility (Clare *et al*, 2006). The distances between V_0 proteolipids appear to be greater than those found in F-ATPases, which contain a cylinder of evolutionarily related proteolipids. It was hence proposed that these gaps might be filled by lipids (Clare *et al*, 2006) or proteins such as SNAREs (El Far and Seagar, 2011). Furthermore, V_0 proteolipids can form inducible pores that translocate polar molecules when reconstituted into liposomes (Morel *et al*, 2001; Peters *et al*, 2001). In light of our observations and the arguments above, our preferred working hypothesis is that the proteolipid cylinder undergoes conformational changes as an entity and that these can be influenced by SNAREs as well as by interactions between the proteolipid cylinder subunits. Several features of the single substitutions obtained in our screen are consistent with this hypothesis. First, substitutions impeding vacuole fusion concentrate within the cytosolic half of the hydrophobic core of the bilayer, close to the interfaces of two ring subunits. This renders it unlikely that they might

influence direct interactions of proteolipids with other fusion-relevant proteins. Second, three independent random mutageneses yielded mutations in similar positions for all three proteolipids. Third, a substitution in only a single cylinder subunit affects fusion. If proteolipids underwent a concerted conformational change even a buried mutation in a single subunit could influence the entire cylinder. Fourth, substitutions in different cylinder subunits affect the same kinetic stage, the transition from *trans*-SNARE pairing to lipid mixing. Finally, several substitutions in different subunits have a similar effect, they stabilize V_1 on V_0. Thus, the contribution of the proteolipid cylinder to fusion does not implicate only one specific subunit but rather the ensemble.

The positioning of the inactivating substitutions should be evaluated in the light of existing models of fusion in order to derive a working hypothesis. A first possibility is to consider effects of the proteolipid cylinder on membrane curvature, which current hypotheses on membrane fusion emphasize as a major factor (Chernomordik *et al*, 2006; Chernomordik and Kozlov, 2008; McMahon *et al*, 2010). Proteins triggering fusion, such as the exocytic calcium sensor synaptotagmin or Doc2 change local membrane curvature by shallow insertions into membranes. Similarly, proteolipids might influence membrane curvature in their vicinity in a way that promotes lipid transition. Recent results on the evolutionarily related proteolipids from F-ATPases are interesting in this regard because F_o proteolipids can locally deform the lipid bilayer. This became apparent by lipid bridges protruding from the membrane between two F_o proteolipid cylinders in proximity. The membrane deformations generate a lipid-dependent attractive force between proteolipid cylinders (Casuso *et al*, 2010). V_0 proteolipids, which are structurally related to F_o proteolipids and share significant sequence similarity with them, might similarly perturb local lipid structure.

Also pore models of fusion offer an aspect that is consistent with our results. One version of these models suggests that membrane-integral protein domains surrounding the fusion site laterally dissociate to form a lipid/protein hybrid structure that promotes lipid transition and expansion of the fusion pore (Lindau and Almers, 1995; Peters *et al*, 2001; Zimmerberg, 2001; Jahn and Grubmüller, 2002; Jackson and Chapman, 2008; Yoon and Shin, 2009). When applied to proteolipids, such a mechanism would require partial dissociation of at least one subunit interface of the proteolipid ring. This might be favoured by the conformational flexibility and the wide spacing of V_0 proteolipids, which was proposed to be due to intercalated lipids (Clare *et al*, 2006). In agreement with a subunit dissociation model, we found that covalent linkage of Vma3p and Vma16p strongly inhibits vacuole fusion; that most fusion-relevant residues are oriented towards the interfaces between proteolipid subunits; and that vma16^{F190Y}, which results in the strongest fusion defect, changes the oligomerization pattern of the proteolipids in SDS–PAGE, suggesting that the subunit interactions are altered. This might interfere with cooperative conformational rearrangements across the proteolipid ring that might be necessary for efficient fusion.

Most proteinaceous pore or fence models postulate a ring-like structure in both fusion partners, although also unilateral ring-like assemblies have been proposed (Lindau and Almers, 1995; Jahn and Grubmüller, 2002). V_0 sectors from two fusing vacuoles can associate, which led to the proposal that they might juxtapose two proteolipid cylinders, creating a gap-junction-like structure as postulated in pore models of fusion (Lindau and Almers, 1995; Almers, 2001; Peters *et al*, 2001). Since subsequent functional tests indicated that a complete V_0 sector is required in only one of the two fusion partners in order to promote fusion (Baars *et al*, 2007; Takeda *et al*, 2008) these V_0–V_0 associations do probably not represent a functionally relevant unit. Since vacuole fusion is a homotypic event they could be mediated by vacuolar v- and t-SNAREs, which both interact with V_0 (Peters *et al*, 2001; Takeda *et al*, 2008) and might thereby hold two V_0 sectors in association. The pore assembly might be heterogeneous, with V_0 in one fusion partner and transmembrane domains of other fusion factors in the opposing membrane. Good candidates for such proteins would be the SNAREs themselves since mutations in their transmembrane anchors can influence fusion (McNew *et al*, 2000; Langosch *et al*, 2007). For exocytic SNAREs, inactivating mutations in the transmembrane anchors were found in positions that are consistent with an arrangement of these anchors in a circular structure (Han *et al*, 2004; Jackson and Chapman, 2008). Thus, despite the asymmetric participation of V_0, a pore-like protein/lipid assembly remains a possibility.

We favour an interpretation of the asymmetrical requirement for V_0 in the light of unilateral pore models in which the bilayer in only one membrane would have to be destabilized by V_0 (Lindau and Almers, 1995). The proteolipid cylinder promotes fusion of yeast vacuoles at a stage subsequent to or independent of *trans*-SNARE pairing. It acts as an entity, requiring a specific conformation that depends on proteolipid residues buried within the bilayer, that is not attainable if the cylinder subunits are covalently linked and that requires the presence of the v-SNARE Nyv1. We propose that partial dissociation of at least two proteolipid subunits creates a hydrophobic cleft that promotes the reorientation of lipids and the induction of hemifusion (Figure 9). The driving force

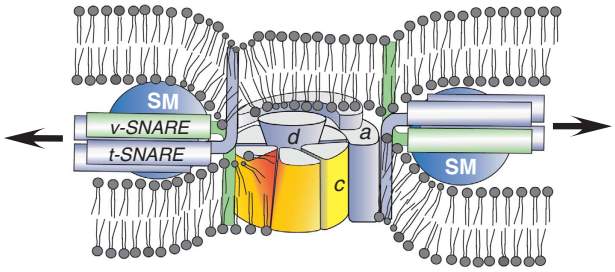


Figure 9 Working hypothesis. We assume that hydrophobic surfaces in the proteolipid cylinder initiate lipid reorientation and hemifusion. They are exposed by a transient conformational change, illustrated in the form of an orange crevice between the yellow proteolipids (collectively designated as 'c'). V_0 subunit e is not shown because there are no data implicating it in fusion. The hydrophobic crevice could be invaded by lipid acyl chains, changing lipid orientation and bilayer curvature. A lipidic fusion pore could then form at the periphery of the cylinder and expand laterally. The conformational change of proteolipids may be promoted by SNAREs. Association with the SM protein and Rab-GTPase associated tether factors might restrict the rotational freedom of SNAREs and arrest their complex in a tense, twisted conformation (Pieren *et al*, 2010), which should exert mechanical strain on the bilayer. In addition, the size of the SNARE/SM complex should drive it away from a site of close membrane apposition or hemifusion, thus creating lateral tension on the fusion site (Mayer, 2001; Rizo *et al*, 2006).

for this transition could be provided by the formation of *trans*-SNARE complexes, which might transduce some of the mechanical strain produced by their formation to V_0. SNAREs might create strain in several ways: either by being twisted, favoured by their association with large protein complexes that might restrict the rotational freedom of their hydrophilic domains, such as SM proteins and tether complexes (Pieren *et al*, 2010); or by the size of SNARE complexes and their associated proteins. Their size should drive them away from the fusion site, which requires a tight apposition of the membranes that might disfavour the presence of large proteins (Mayer, 2001; Rizo *et al*, 2006). Our current working model differs from previous pore models, which postulated proteinaceous rings to provide amphiphilic tracks promoting the movement of both lipid leaflets between the membranes (Lindau and Almers, 1995). We assign to the proteolipids a function that lies mainly in modulating lipid orientation and curvature. In this sense, our working model is related to hypotheses based on curvature generation by membrane-inserting peptides. However, it relies on integral membrane proteins rather than on peripheral proteins as a means to influence lipid curvature. Hemifusion induced in this way could evolve into a lipidic fusion pore that could remain entirely outside the cylinder or form at its outer surface. It could expand without the need for dissociating the entire ring structure. Future experiments will further test how well an influence on lipid orientation can explain the activity of V_0 in fusion. They will be directed towards the purification of proteolipids, their co-reconstitution with SNAREs and the analysis of their structure.

Materials and methods

Yeast strains used are listed in Table I. Methods and reagents not found here are described in Supplementary data.

Library construction

VMA genes were randomly mutagenized by error-prone PCR using the GeneMorph Random Mutagenesis Kit (Stratagene). The mutation rate was set to about 2 per 100 bp by adjusting the template concentration to 5–25 pg and running the mutagenic PCR for 30 cycles. As template, we used the corresponding VMA gene on the pRS416 backbone. Mutagenized amplification products were blunt end cloned into TOPO XL and excised using corresponding restriction enzymes (see Supplementary Table S4 for primer sequences and restriction sites). Excised VMA variants were cloned into pRS416 downstream of their endogenous promoter. The plasmid libraries were transformed into corresponding BJ3505 vma deletion strains using the lithium acetate method with 1 h heat shock times. Transformants were scraped off plates, pooled and stored at −80 °C.

Screening procedure and vacuolar staining with FM4-64

Libraries were spread on plates containing HC-URA 50 mM HEPES pH 7.5 to make sure that only mutants with correctly assembled V-ATPase survive and proceed to the next step of screening. Surviving colonies were transferred into deep 96-well plates and grown in YPD 50 mM MES pH 5.5. To visualize vacuolar membranes, *in vivo* cells were stained with the lipophilic vital dye *N*-(3-triethylammoniumpropyl)-4-(*p*-diethylaminophenylhexatrienyl)-pyridinium dibromide (FM4-64) (Vida and Emr, 1995): Cells were grown overnight at 30 °C to dense cultures in 96-well plates. After 1/1000 dilution, cells were grown overnight at 27 °C to logarithmic phase (OD_{600} <1). In all, 15 μM FM4-64 was added from a 4-mM stock solution in 70% EtOH. Cells were incubated for 1 h at 27 °C, harvested (2 min, 3000 g), washed in fresh medium, resuspended in YPD 0.5 mM MES pH5.5 at $OD_{600} = 0.4$, and shaken for 2–3 h at 27 °C. For microscopy, cells were transferred to 96-well glass bottom plates (NUNC) and analysed on a spinning disc fluorescence microscope using a 488-nm excitation laser and a ×100 objective. Exposure times did not exceed 100 ms. Images were processed with ImageJ.

ACMA in vitro proton pumping assay

To assess pumping activity in isolated vacuoles, we used a fluorescence quenching assay. ACMA is a acidophilic fluorescent agent that accumulates in acidic environments like the vacuole upon incubation with ATP (Casadio, 1991). In all, 300 μl fusion reactions were assembled without the ATP regenerating system. ACMA was added from a 5-mM stock solution (in DMSO) to a final concentration of 16.6 μM. Reactions were split into three 90 μl aliquots in wells of non-coated black 96-well plates (no. 237105, NUNC). Concanamycin A was added to some samples to a final concentration of 0.4 μM and all reactions are allowed to equilibrate at 27 °C for 10 min. Fluorescence change was measured with a SpectraMax GeminiXS fluorescent plate reader (Molecular Devices) at 27 °C, 410 nm excitation and 490 nm emission. Measurements were taken every 11 s in three time slots. In slot 1 (2 min), the initial fluorescence was acquired. Then, an ATP regenerating system was added to all samples, either containing ATP (+ATP) or lacking ATP (−ATP). The fluorescence decrease due to quenching of ACMA fluorescence was monitored in time slot 2 (3 min). At the end of the reaction, the membrane gradients were dissipated with 60 μM FCCP and recovery of the initial fluorescence level was monitored in time slot 3 (3 min).

Trans-SNARE assay

In all, 1 ml fusion reactions (containing 400 μg vacuoles) with cytosol (1 mg/ml) were incubated for 30 min at 27 °C. The reactions

Table I Yeast strains used in this study

Strain	Genotype	Source or reference
BJ3505	Mat a pep4::HIS3 prb1-Δ1.6R lys2-208 trp1-Δ101 ura3-52 gal2 can	Jones *et al* (1982)
DKY6281	Mat á leu2-3 leu2-112 ura 3–52 his3-Δ200 trpl-Δ901 lys2-801 suc2-Δ9 pho8::TRP1	Haas *et al* (1994)
BJ3505 Δvma3	BJ3505; vma3::TRP1	Baars *et al* (2007)
BJ3505 Δvma11	BJ3505; vma11::TRP1	Baars *et al* (2007)
BY4741 Δvma16 Δpep4	Mat a his3Δ leu2Δ met15Δ ura3Δ vma16::natNT2, pep4::kanMX4	Takeda *et al* (2008)
DKY6281 Δvma3	DKY6281; Δvma3::kanMX	Bayer *et al* (2003)
DKY6281 Δvma11	DKY6281; Δvma11::kanMX	Bayer *et al* (2003)
DKY6281 Δvma16	DKY6281; Δvma16::kanMX	Bayer *et al* (2003)
BJ3505 Δvma3 VAM3–VSV	BJ3505; vma3::TRP, VAM3::VSV	This study
BJ3505 Δvma11 VAM3–VSV	BJ3505; vma11::TRP, VAM3::VSV	This study
BJ3505 Δvma3 NYV1–HA	BJ3505; vma3::TRP, NYV1::3xHA	This study
BJ3505 Δvma11 NYV1–HA	BJ3505; vma11::TRP, NYV1::3xHA	This study
BJ3505 Δnyv1	BJ3505; nyv1::TRP1	Nichols *et al* (1997)

were stopped by addition of 5 mM EDTA and chilling the samples on ice. Vacuoles were solubilized in 1 ml lysis buffer (0.8% Triton X-100, 150 mM NaCl, 10% glycerol 0.75 mg/ml BSA, 1 mM PMSF, 3 mM EDTA, 2 mM DTT, 1 × PIC in 20 mM HEPES pH 7.4) by gentle shaking (5 min, 4 °C) and centrifuged (4 min, 14 000 g). The solubilisate was transferred to another tube. In all, 2% of the volume was kept as load control. In all, 14 μg of anti-HA antibody (Covance) and 30 μl of protein G-agarose (Roche), pre-equilibrated in lysis buffer, were added to each sample. After 2 h of end-over-end rotation at 4 °C, the protein G-agarose was washed three times with washing buffer (0.2% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM PMSF, 1 × PIC in 20 mM HEPES pH 7.4). In all, 16 μl SDS sample buffer was added and aliquots were analysed by SDS–PAGE and western blot.

Statistical analysis

Values from at least three independent experiments were averaged. Error bars represent the standard deviation of the mean. Samples showing significant differences ($P < 0.01$, Student's *t*-test) relative to the wild type are indicated with an asterisk.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: BS, AM, JI and OM contributed to the experimental design; AM and BS contributed to the writing of the manuscript; and BS and JI contributed to the execution of the experiments.

Conflict of interest

The authors declare that they have no conflict of interest.

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