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

Bernd Strasser, Justyna Iwaszkiewicz, Olivier Michielin and Andreas Mayer

Corresponding author: Andreas Mayer, University of Lausanne

Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
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</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>08 April 2011</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>12 May 2011</td>
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<tr>
<td>Revision received</td>
<td>05 July 2011</td>
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<tr>
<td>Editorial Decision</td>
<td>16 August 2011</td>
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<tr>
<td>Revision received</td>
<td>19 August 2011</td>
</tr>
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<td>Accepted</td>
<td>19 August 2011</td>
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 12 May 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, while referee 2 is more critical and thinks that deeper mechanistic understanding of how V0 acts at the molecular level would be needed, the other two referees are very positive and would support publication here, referee 1 after some revision. After looking into the case in depth and given the strong positive vote by referees 1 and 3, I have come to the conclusion to follow the majority vote in this case. I would thus like to invite you to submit a revised version of the manuscript, in which you need to address the referees' concerns in an adequate manner. I will not ask you to develop the study further mechanistically, but I would like to ask you to address or respond to the remaining points raised by referee 2. Please do not hesitate to get back to me if you wish to discuss any aspect of the revision further.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the
conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS

Referee #1

The V-ATPase is a ubiquitous multi-subunit proton pump that acidifies intracellular membrane compartments. A variety of data suggests that subunits of the membrane sector (V0) also play a direct role in membrane fusion. However this is a difficult area of study as genetic deletion of V0 sector subunits can perturb the proton pump, thus compromising cell viability. To try and resolve this issue by genetically separating the two functions, the authors have carried out random mutagenesis of the proteolipid subunits in yeast (Vma3, Vma11 & Vma16) to identify mutations affecting vacuole fusion, but not acidification. In my view they have succeeded, however the following points must be addressed.

1. The manuscript needs careful revision throughout. The figures are complex and not always self-explanatory. Too much going back and forth between the main text, legends and figures is required to fully grasp the data. Furthermore there are a several errors in the legends and text references to figures.
   - Fig 4f illustrates data with the 16-3-HA construct, but the abbreviation is not explained in the legend.
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2. At the bottom of page 12 the authors state "Proteolipids should act subsequent to or independently of SNARE activation........but we find it hard to envisage how single proteolipid substitutions within the bilayer should influence SNARE complex zippering". Yet the vma11 L141V mutation reduces SNARE pairing by 50% (Fig 7 and page 10) and this mutation is located in the 4th transmembrane region of vma11 (Fig 5). The authors need to clear up this discrepancy and discuss the effects of the L141V mutation.

3. An important finding in this study is that deletion of the v-SNARE Nyv1p appears to tighten the interaction between the V1 and V0 sectors of the V-ATPase, in a similar way to the proteolipid point mutations. This result suggests that manipulations which increase v-SNARE availability might destabilize the V1/V0 complex. The authors should determine whether SNARE-priming (dissociating cis SNARE complexes to increment unpaired Nyv1p) reduces V1 binding to V0 ? This experiment would strengthen their arguments.

4. From Fig 8D, the authors conclude that the 16-3-HA fusion construct does not form high molecular weight oligomers (page 11). However the tandem monomer migrates much more slowly than Rec16-HA monomer, thus 16-3-HA oligomers (migrating at >60kDa) would not be visible anyway in the illustration of the gel provided. An illustration of a gel with the capacity to resolve the 16-3-HA oligomers should be provided.
5. The data is consistent with the view that the proteolipid point mutations are effective at steps proximal to membrane fusion, but do not yet give any clear indication of the mechanisms by which proteolipids act. The discussion focuses principally on the hypothesis that the mutations (and v-SNARE binding) affect the conformation of the proteolipid cylinder leading to a modification in lipid interaction and membrane curvature. The hypothesis is difficult to visualize and would benefit from a better schema / cartoon. Supplementary Fig 5 is not satisfactory as the differences between panels A and B are not immediately obvious. The v-SNARE / proteolipid interaction is hardly visible (hidden by the lipids), nor is the hydrophobic cleft. Furthermore as membrane curvature is an important factor a second SNARE complex could be added on the right to portray radial symmetry.

6. The authors place far too much emphasis on a single hypothesis, the discussion on the mechanistics by which proteolipids influence fusion should be broader.

Minor points

The title is not appropriate. It suggests that the proteolipid cylinder is acting initially on lipid mixing which then has consequences on fusion. In fact lipid mixing is probably just concomitant with fusion. They could consider "...lipid mixing and SNARE-dependent fusion...".

Referee #2

This manuscript addresses the potential role for the core proteolipid ring of the membrane-bound V0 segment of the yeast V-ATPase in vacuole-vacuole fusion. The authors randomly mutagenize all three of the yeast proteolipid genes and select for mutants that retain sufficient V-ATPase activity to grow at pH 7.5, then screen these mutant strains for vacuolar fragmentation, as evidence of defective vacuole-vacuole fusion. (This is a laborious screen that is carefully done.) Point mutations in all three proteolipid subunits were isolated that supported at least some vacuolar acidification, but were defective in lipid and content mixing in vacuole-vacuole fusion, suggesting that they affect a step prior to hemifusion. Most of the proteolipid mutations allow trans-SNARE pairing, further narrowing the step that is affected in fusion. In the discussion, the authors propose a detailed model for the role of the proteolipid cylinder in membrane fusion, placing it at the stage between SNARE pairing and lipid mixing, and arguing that one potential role of the proteolipids is SNARE-dependent deformation of the membrane so lipid mixing can occur.

Although the experiments are well-done for the most part, the data do not support the proposed mechanistic models, and this significantly weakens the paper. Previous data have supported a role for the V0 complex, primarily the a subunit, in membrane fusion in this and other systems, so it is not surprising that mutations in the proteolipids can affect fusion; it is surprising that the authors present a model in Supplementary Fig. 5 that does not appear to include any role for the a subunits. The authors argue that the new point mutations alter/stabilize proteolipid structure in a manner that prevents lipid mixing at a post-SNARE pairing step, but the actual data for this is quite weak. Fig. 8D, showing a higher molecular weight species in the VM 1A16 F190Y mutant, contributes very little to the argument, since the identity of the higher species is very unclear. The proteolipid laddering seen elsewhere and referred to in the text consists of multimers of proteolipids; the species seen here are too small for multimers containing Vma16 and could instead be a misfolded population. The increased assembly of V1 subunits with the mutant V0 subunits seen in Fig. 8 A and B does suggest a conformational change in V0, but also suggests an alternative explanation for the fusion phenotype that is not considered. The increase in assembly with V1 in the point mutants is quite substantial and inversely proportional to the decrease in lipid mixing (Fig. 6), so it is possible that reduced fusion comes from having fewer unbound V0 sectors available for participation in the fusion process. The presence of the bulky V1 sectors bound to the V0 sectors could also influence the partially to fully
zipped trans-SNARE transition, a possibility the authors dismiss as unlikely in the Discussion. No direct data is presented in support of the working hypothesis proposed on p. 14, which suggests that after binding to SNAREs, the proteolipids influence membrane curvature, deforming the lipid bilayer. Further experiments to test such a model would provide the type of mechanistic insights that would strengthen the paper.

Minor points:

1) Although it is likely that the proteolipid point mutants do retain proton pumping capability, based on the growth phenotypes, their level of activity may be greatly overestimated in Fig. 4 E and F because the very rapid decrease in the wild-type sample indicates that the assay is saturated.

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3) The referencing of the paper has a number of problems. For example, Cohen et al, 1995 is repeatedly cited, but I could not find this article at all. Cohen and Melikyan, 2004 also appears to be cited inappropriately. Some references are incomplete: for example, Di Giovanni et al. has no journal in the reference list.

4) Supplementary Fig. 4 is unnecessary.

Referee #3

In this paper, the role of the V-ATPase V0 sector in vacuole fusion has been further explored. The authors have designed an elegant mutagenesis and screening procedure to generate mutants in the proteolipid subunits VMA3 11 and 16 that did not affect the proton pump activity but cause vacuole fragmentation. From this analysis, they recovered alleles, which do not affect proton translocation, SNARE activation and trans-SNARE pairing, but which interfere with fusion, using assays that measure mixing of lipid or content. They report that the residues of interest are found in each of the proteolipid subunits and that they seem to concentrate within the membrane in regions at the interface between subunits. Finally, they report that interactions of V0 and V1 are stabilized by the substitutions of interest as they are by deletion of the vacuolar v-SNARE Nyv1, and that a fusion of two proteolipid subunits inhibits fusion. Authors conclude that Nyv1 and proteolipid mutations have similar effects on the conformation of V0, and that such a conformational change plays a role in pore formation, perhaps by promoting lipid reorientation.

I find the paper very interesting. It is also very well done. The data are of high quality and the experiments are convincing. The possible role of V0 in fusion has been proposed some time ago by the same group, but has been a matter of debate since. Evidence from several groups is now accumulating that supports this notion, and recent papers have reported the existence of V0-SNARE interactions. However, the mechanism remains mysterious, and clearly the next step (what is V0 actually doing?) will be really exciting. But this paper already provides solid data that a conformational change in V0 plays a role in controlling fusion, in a process involving Nyv1. I recommend publication without hesitation.

1st Revision - authors’ response 05 July 2011

We thank the reviewers for their comments and questions, which helped us to improve our model, made our argumentation more precise and the discussion more balanced.

Referee #1
The V-ATPase is a ubiquitous multi-subunit proton pump that acidifies intracellular membrane compartments. A variety of data suggests that subunits of the membrane sector (V0) also play a direct role in membrane fusion. However this is a difficult area of study as genetic deletion of V0 sector subunits can perturb the proton pump, thus compromising cell viability. To try and resolve this issue by genetically separating the two functions, the authors have carried out random mutagenesis of the proteolipid subunits in yeast (Vma3, Vma11 & Vma16) to identify mutations affecting vacuole fusion, but not acidification. In my view they have succeeded, however the following points must be addressed.

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We agree with the reviewer that the text is not sufficiently clear in this regard. That several mutations affect lipid and content mixing while allowing normal trans-SNARE formation shows that proteolipids make a contribution to the actual fusion event that is separable from docking. It does not exclude that they in addition influence the earlier step of trans-SNARE pairing and, as the reviewer correctly remarks, vma11L141V actually shows this. We have changed the text on pages 9 and 10 and in the discussion (page 13) accordingly, stressing this fact and clearly distinguishing these two effects.

3. An important finding in this study is that deletion of the v-SNARE Nyv1p appears to tighten the interaction between the V1 and V0 sectors of the V-ATPase, in a similar way to the proteolipid point mutations. This result suggests that manipulations which increase v-SNARE availability might destabilize the V1/V0 complex. The authors should determine whether SNARE-priming (dissociating cis SNARE complexes to increment unpaired Nyv1p) reduces V1 binding to V0 ?

This is a good idea which we have tried. The experiment did not show a clear effect of Sec18p/NSF activity on V1 association with vacuoles. This suggest that Sec18 does not actively disassemble V1/V0 but it does not contradict an effect of Nyv1 on this interaction. We assume that free Nyv1 can associate with the pool of free V0 sectors, thereby shifting the equilibrium of V-ATPase dissociation and reassociation towards the dissociated state. In line with this we observed that overexpression of Nyv1 reduces V1 association with the membranes, providing further support of the notion that Nyv1 destabilizes the V-ATPase. This data is now included in Fig. 7C.

Based on several, partly unpublished observations, we have not expected a detectable effect of Sec18 activity on V1 dissociation. First, the V-ATPase is at least 10x more abundant than the t-SNAREs on the vacuoles and the amounts of V0 can be lowered 20-fold before affecting fusion. Thus, a small fraction of the V0 pool suffices to satisfy the requirements for fusion. Second, Nyv1 is significantly more abundant than the other vacuolar SNAREs and a large fraction of Nyv1 does not
exist in a stable complex with the other vacuolar SNAREs on freshly prepared vacuoles. Accordingly, an up to 5-fold lower fraction of the Nyv1 pool is recovered in SNARE complexes as compared to the other vacuolar SNAREs (see for example Collins and Wickner, 2005; Wang et al., 2003). In other words, the free pool of Nyv1 on isolated vacuoles could hardly be increased further by priming. In line with this, Wickner and colleagues found that overexpressing Nyv1 does not increase trans-SNARE pairing and concluded that the amounts of free Nyv1 are not limiting for fusion.

4. From Fig 8D, the authors conclude that the 16-3-HA fusion construct does not form high molecular weight oligomers (page 11). However the tandem monomer migrates much more slowly than Rec16-HA monomer, thus 16-3-HA oligomers (migrating at >60kDa) would not be visible anyway in the illustration of the gel provided. An illustration of a gel with the capacity to resolve the 16-3-HA oligomers should be provided.

The expression in the text is ambiguous. We intended to say that the higher migrating bands observed for Vma16HA are absent in a strain expressing the Vma16-3HA fusion protein, i.e. we used the Vma16-3HA only as a control for the specificity of the Vma16HA bands. We did not want to say that the Vma16-3HA fusion could or could not make any oligomers. For this fusion construct higher MW associations would be difficult to analyze due to technical limitations. In order to detect proteolipids by Western blot we have to push the sensitivity quite far, leading to the appearance of non-specific bands above 35 kDa which overlap with the region where we might expect Vma16-3 oligomers. We present the complete gel that gave rise to Fig. 8D below in order to illustrate this. An alternative is that Vma16-3 containing oligomers might be less stable due to a conformational influence of the Vma16-3 fusion on the proteolipid cylinder. Since there is, at present, no possibility to distinguish these two alternatives we did not make an argument out of it and used the band pattern of the Vma16-3 strain only as a specificity control for the antibody. We have now changed the respective paragraph in the results in order to make this intention more evident.

5. The data is consistent with the view that the proteolipid point mutations are effective at steps proximal to membrane fusion, but do not yet give any clear indication of the mechanisms by which proteolipids act. The discussion focuses principally on the hypothesis that the mutations (and v-SNARE binding) affect the conformation of the proteolipid cylinder leading to a modification in lipid interaction and membrane curvature. The hypothesis is difficult to visualize and would benefit from a better schema / cartoon. Supplementary Fig 5 is not satisfactory as the differences between panels A and B are not immediately obvious. The v-SNARE / proteolipid interaction is hardly visible (hidden by the lipids), nor is the hydrophobic cleft. Furthermore as membrane curvature is an important factor a second SNARE complex could be added on the right to portray radial symmetry.

We have revised the figure, trying to make the crucial changes more evident. In particular, we have introduced a 3D-representation of the proteolipid cylinder which now clearly shows a cleft, coloured in red. Furthermore, we have taken care to keep the SNAREs in contact with the proteolipid cylinder and the a subunit, thus incorporating published observations from other systems. We have
abandoned panel A because it is not so easily reconciled with the presence of the d subunit in the central cavity of the cylinder. Our remaining working model is now presented as Fig. 10.

6. The authors place far too much emphasis on a single hypothesis, the discussion on the mechanisms by which proteolipids influence fusion should be broader.

We did consider several alternatives, such as the fact that SNARE availability or pairing might be influenced, that SNARE concentration around a fusion site might be influenced, or SNARE zippering (which cannot be directly measured in any physiological membrane so far). We have now added the possibility that the point mutations might specifically influence proteolipid-v-SNARE interactions, which are important in the neuronal system and that they might influence trans-SNARE pairing, and that they might influence SNARE zippering or deplete the pool of free V0. We briefly mention the aspects of our data that are inconsistent with these views and then focus on the cleft hypothesis which accommodates all our observations so far. We feel that our data provide no strong reasons to invoke further scenarios (at least at present). We’d be open to discuss further possibilities that the reviewer might see and that are equally consistent with our data.

Minor points

The title is not appropriate. It suggests that the proteolipid cylinder is acting initially on lipid mixing which then has consequences on fusion. In fact lipid mixing is probably just concomitant with fusion. They could consider "...lipid mixing and SNARE-dependent fusion...." We consider lipid mixing as a part of fusion and hence find the proposed alternative not optimal. We realize, however, that it is better to be more precise and therefore changed the title to "...promotes the lipid mixing stage of SNARE-dependent fusion...."

page 3, line 9 : and microtubules
page 19, line 7 : dextran
page 27 : Takeda et al reference should be Ungermann C, not Christian U
Use capitals throughout when referring to figure panels in the text.

These points have been corrected.

Referee #3

This manuscript addresses the potential role for the core proteolipid ring of the membrane-bound V0 segment of the yeast V-ATPase in vacuole-vacuole fusion. The authors randomly mutagenize all three of the yeast proteolipid genes and select for mutants that retain sufficient V-ATPase activity to grow at pH 7.5, then screen these mutant strains for vacuolar fragmentation, as evidence of defective vacuole-vacuole fusion. (This is a laborious screen that is carefully done.) Point mutations in all three proteolipid subunits were isolated that supported at least some vacuolar acidification, but were defective in lipid and content mixing in vacuole-vacuole fusion, suggesting that they affect a step prior to hemifusion. Most of the proteolipid mutations allow trans-SNARE pairing, further narrowing the step that is affected in fusion. In the discussion, the authors propose a detailed model for the role of the proteolipid cylinder in membrane fusion, placing it at the stage between SNARE pairing and lipid mixing, and arguing that one potential role of the proteolipids is SNARE-dependent deformation of the membrane so lipid mixing can occur.

Although the experiments are well-done for the most part, the data do not support the proposed mechanistic models, and this significantly weakens the paper. Previous data have supported a role for the V0 complex, primarily the a subunit, in membrane fusion in this and other systems, so it is not surprising that mutations in the proteolipids can affect fusion; it is surprising that the authors present a model in Supplementary Fig. 5 that does not appear to include any role for the a subunits. The authors argue that the new point mutations alter/stabilize proteolipid structure in a manner that prevents lipid mixing at a post-SNARE pairing step, but the actual data for this is quite weak.
The reviewer does not specify why the evidence we provide should be considered as weak. We present point mutants that show normal trans-SNARE pairing but reduced lipid and content mixing, thus separating these events. The simultaneous resolution of these three events cannot be obtained in any other physiological membrane system so far. Therefore, our characterization goes further than possible in other systems and we consider this as a strong point of the paper rather than weak evidence.

One could speculate that partially zipped trans-SNARE complexes might exist (they could not be directly shown during a fusion reaction so far) and that the point mutants could arrest fusion because they prevent full zippering of SNAREs. If so, however, this could only happen by a conformational change of V0 that is transmitted to the SNAREs. Also in that case proteolipids would remain an integral part of the fusion machinery and our assignment of its function to the lipid mixing stage would remain correct.

A conformational change of V0 is the only possible explanation of the effect of proteolipid point mutations on fusion since the affected residues are deeply embedded in the bilayer where they cannot directly interact with the soluble V1 sector, nor with the SNARE domains. Furthermore, covalent linkage of proteolipids blocks fusion. This linkage connects hydrophilic ends of two proteolipids on the lumenal face of the membrane whereas fusion must be initiated on its cytosolic face. Also here we see a conformational change of the proteolipids as the only possible explanation. Therefore, we consider our conclusion of a conformational change as necessary and justified. This reviewer gives no indication why our conclusion of a conformational change of proteolipids should be insufficiently supported and which alternative explanations could be given. In addition, in a subsequent comment this reviewer states that “The increased assembly of V1 subunits with the mutant V0 subunits seen in Fig. 8 A and B does suggest a conformational change in V0”. We would be happy to consider alternative explanations of our data if the reviewer provided them.

Fig. 8D: showing a higher molecular weight species in the VMA16 F190Y mutant, contributes very little to the argument, since the identity of the higher species is very unclear. The proteolipid laddering seen elsewhere and referred to in the text consists of multimers of proteolipids; the species seen here are too small for multimers containing Vma16 and could instead be a misfolded population.

The running behaviour can be understood from the sequence of the fusion protein, which was not available to this reviewer. Vma3p has 16 kDa, Vma16pHA has 23 kDa. Tracing how the Vma16-Vma3HA fusion was made by the Stevens lab it becomes apparent that Vma16 and Vma3 are joined by a triple HA tag plus additional spacer sequences, adding 4 kDa and bringing the MW of the fusion protein to 42 kDa. However, this fusion protein runs below the 35 kDa marker, i.e. 7 kDa faster than expected from its MW. This suggests that also a non-covalent Vma16/Vma3 oligomer might run faster than expected. In line with this, the first oligomer band of Vma16 alone runs 8 kDa lower (approx. 31 kDa) than expected from the sum of the molecular weights (39 kDa) and at a distance of approx. 4 kDa relative to the Vma16-Vma3HA fusion protein, i.e. with a shift representing the missing covalent linker.

It should be kept in mind that the apparent MW of a complex in SDS-PAGE need not be equivalent to sum of the molecular weights of two subunits. Substantial deviations can occur, depending on the folding and detergent loading of the proteins, both of which may be different for a (at least partially folded) complex relative to its dissociated subunits, which may be more completely unfolded and do not have interaction surfaces that may be shielded from detergent access.

The increased assembly of V1 subunits with the mutant V0 subunits seen in Fig. 8 A and B does suggest a conformational change in V0, but also suggests an alternative explanation for the fusion phenotype that is not considered. The increase in assembly with V1 in the point mutants is quite substantial and inversely proportional to the decrease in lipid mixing (Fig. 6), so it is possible that reduced fusion comes from having fewer unbound V0 sectors available for participation in the fusion process. The presence of the bulky V1 sectors bound to the V0 sectors could also influence the partially to fully zipped trans-SNARE transition, a possibility the authors dismiss as unlikely in the Discussion.
We consider it as unlikely that the amount of free V0 becomes limiting because on isolated vacuoles less than 20% of V0 is associated with V1. During the first 10 min of an in vitro fusion reaction 30-50% of V1V0 holoenzymes dissociate. Thus, there must be an ample supply of free V0 sectors. In addition, we know that the abundance of V0 on vacuoles can be reduced by 95% without affecting fusion. The latter observation is consistent with the fact that V0 is at least 10x more abundant than vacuolar t-SNAREs.

Fusion could also not be inhibited by V1 stabilization and a resulting sterical hindrance of full SNARE zippering because, we would expect not to see any trans-SNARE pairing at all in this case, simply because V1 would be too big to allow any contact of a V-ATPase-associated SNARE with another SNARE in trans. Even two SNARE domains could not span the distance. Since we observe normal trans-SNARE pairing in most proteolipid mutants we dismiss this hypothesis. This aspect is now addressed more extensively in the Discussion.

No direct data is presented in support of the working hypothesis proposed on p. 14, which suggests that after binding to SNAREs, the proteolipids influence membrane curvature, deforming the lipid bilayer. Further experiments to test such a model would provide the type of mechanistic insights that would strengthen the paper.

We agree, such evidence would be a tremendous step forward. Obtaining it will be a study of its own, however, requiring a variety of experimental approaches and controls that would go beyond the scope and size limits of this manuscript.

Minor points:

1) Although it is likely that the proteolipid point mutants do retain proton pumping capability, based on the growth phenotypes, their level of activity may be greatly overestimated in Fig. 4 E and F because the very rapid decrease in the wild-type sample indicates that the assay is saturated.

In Fig. 4 E the time between addition of ATP and closure of the photometer had perhaps been too long so that the initial decline was missed. Fig. 4E was therefore exchanged for a better experiment (now Fig. 3E). We want to stress that the curves in Fig. 4 appear relatively steep due to the way we plot them. This does not reflect a lack of measuring points, however, since we show only every third recorded timepoint, which allows a better representation of the symbols. We have now changed the representation of the data in Fig. 4F, showing more data points in the steeper part of the curve (now Fig. 3F).

We have, of course, explored the properties of the assay before using it. Our titrations with different V-ATPase inhibitors indicated that the assay as we perform it responds to these inhibitors in the expected concentration ranges and hence is sufficiently sensitive to detect also smaller variations in V-ATPase activity. As an example, we performed a titration of archazolid A with the assay as we use it. The measured activities yielded an IC50 of approx. 8 nM, in agreement with the published IC50 of 6.6 nM for archazolid A on yeast vacuoles (see Wieczorek and coworkers, JBC 285, 3830, Tab. 3).

2) It is not clear where the VMA3 T32I mutation used throughout the paper came from, since the mutagenesis appeared to give VMA3 T32S (Suppl. Table 1). This point should be clarified.

Vma3T32I is a known mutant that reduces the inhibitor sensitivity of the V-ATPase, which is the reason why we had it since many years. After having identified the Y97j allele, we separated its three substitutions and learned that a moderate fusion defect was linked to T32S. Since we noticed that the existing T32I substitution gave better inhibition of fusion with fewer side effects on pumping we continued to explore the relevance of T32 with this mutant. This explanation has now been added to the text.
3) The referencing of the paper has a number of problems. For example, Cohen et al, 1995 is repeatedly cited, but I could not find this article at all. Cohen and Melikyan, 2004 also appears to be cited inappropriately. Some references are incomplete: for example, Di Giovanni et al. has no journal in the reference list.

These are probably side effects of our latest change in the reference manager software, which created some problems with the library. We have checked the references and eliminated some errors.

4) Supplementary Fig. 4 is unnecessary.

Discussions with colleagues repeatedly show that they are particularly keen on knowing which substitutions from our screen concern conserved residues or regions and might be transferred to higher eukaryotes. Supplementary Fig. 4 serves this purpose and we therefore prefer to keep it, even though it does not present a new result of our research.

Referee #3

In this paper, the role of the V-ATPase V0 sector in vacuole fusion has been further explored. The authors have designed an elegant mutagenesis and screening procedure to generate mutants in the proteolipid subunits VMA3 11 and 16 that did not affect the proton pump activity but cause vacuole fragmentation. From this analysis, they recovered alleles, which do not affect proton translocation, SNARE activation and trans-SNARE pairing, but which interfere with fusion, using assays that measure mixing of lipid or content. They report that the residues of interest are found in each of the proteolipid subunits and that they seem to concentrate within the membrane in regions at the interface between subunits. Finally, they report that interactions of V0 and V1 are stabilized by the substitutions of interest as they are by deletion of the vacuolar v-SNARE Nyv1, and that a fusion of two proteolipid subunits inhibits fusion. Authors conclude that Nyv1 and proteolipid mutations have similar effects on the conformation of V0, and that such a conformational change plays a role in pore formation, perhaps by promoting lipid reorientation.

I find the paper very interesting. It is also very well done. The data are of high quality and the experiments are convincing. The possible role of V0 in fusion has been proposed some time ago by the same group, but has been a matter of debate since. Evidence from several groups is now accumulating that supports this notion, and recent papers have reported the existence of V0-SNARE interactions. However, the mechanism remains mysterious, and clearly the next step (what is V0 actually doing?) will be really exciting. But this paper already provides solid data that a conformational change in V0 plays a role in controlling fusion, in a process involving Nyv1. I recommend publication without hesitation.

We thank this reviewer for sharing our enthusiasm for the topic.

2nd Editorial Decision 16 August 2011

Thank you for sending us your revised manuscript. Let me first of all apologise for the delay in getting back to you with a decision. This was caused by difficulties with the availability of our original referees during the current summer holiday season. In the meantime, referees 1 and 3 have seen the manuscript again and are now fully supportive.

Still, referee 1 puts forward a number of minor points that should be corrected/modified (see below).

Furthermore, there are a number of editorial issues that need further attention. First, please add scale bars together with explanations to all microscopic pictures (fig. 1A-C; 8A).
Second, prior to acceptance of every paper we perform a final check for figures containing lanes of gels that are assembled from cropped lanes. While cropping and pasting may be considered acceptable practices in some cases (please see Rossner and Yamada, JCB 166, 11-15, 2004) there needs to be a proper indication and explanation in all cases where such processing has been performed according to our editorial policies. Please note that it is our standard procedure when images appear like they have been pasted together without proper indication (like a white space or a black line between) or explanation to ask for the original scans (for our records). In the case of the present submission there are a number of panels that do not appear to fully meet these requirements: figure 6A, middle panel. Could you please confirm that in figure 6A, middle panel, all lanes in the horizontal panels come from the same gel and provide us with the original scans. Ideally, it would be better to replace the panels with exposures that do not have such a strong contrast. I am sorry to have to be insistent on this at this late stage. However, we feel that it is in your as well as in the interest of our readers to present high quality figures in the final version of the paper.

Thank you very much for your cooperation. I will then formally accept the manuscript.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS
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Referee #1

There are still a few minor typos / errors that need correcting.
-Page 10, line 9, Figs A-C ?
-Page 13 second paragraph line 11 "The (not She) SM protein....
-In Fig 4 A and B are inverted - A is a side view and B a top view.
-Fig 6, panels are not correctly assigned : should be from A - E, not A - C

Referee #3

I liked the previous version of the paper and feel that the authors have adequately addressed the comments made by the reviewers. I see no reason to further delay publication of this interesting paper.

2nd Revision - authors' response 19 August 2011

We were pleased to see that the referees have been satisfied with the revised version of the manuscript. We have corrected the few labeling and typing errors noted by Referee 1. As demanded by you, scale bars were added to Figs. 1 and 8 and their size is indicated in the figure legends. Concerning the middle panel of Fig. 6, I agree with you, the representation of the blot was not good. I can confirm, however, that the bands are adjacent on a gel and have not been pasted together. The corrected version of Fig. 6 now shows the same data in a less contrast-rich form, so that it becomes evident that they have not been manipulated. The problem was simply that all of our blots are recorded with an infrared fluorescence scanner which has a dynamic range much larger than what can be printed on paper. Thus, the final appearance of the blots depends greatly on the output settings chosen for the conversion of these values into an image. We thank the reviewers, in particular Reviewer 1, for their constructive comments and we want to thank you for the efficient handling of the manuscript and your clear guidance in the interpretation and weighing of the reviewers’ comments.