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Steric Hindrance of SNARE Transmembrane Domain Organization Impairs the Hemifusion-to-Fusion Transition

Massimo D'Agostino, Herre Jelger Risselada and Andreas Mayer

Corresponding author: Andreas Mayer, University of Lausanne

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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 17 March 2016

Thank you very much for the submission of your research manuscript to our editorial office. We have now received the full set of reports from the referees that were asked to assess it.

While the reviewers agree in principle on the interesting nature of the observations reported here, they also feel that the study as it stands is not yet fully conclusive and they point out a number of instances in which the data would need to be strengthened before publication. These relate mostly to technical/experimental issues, but as the detailed reports are pasted below I would prefer not to go into the details of them here.

Given the potential interest of your study and the constructive suggestions of the reviewers on how to improve it, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees must be addressed and their suggestions taken on board.

REFEREE REPORTS

Referee #1:

This study explores mechanisms by which SNAREs mediate fusion of yeast vacuoles. The work presents interesting experimental findings. The authors report that, in contrast to fusion in chromaffin cells, yeast vacuole fusion does not appear to be very sensitive to addition of hydrophilic

tags (small or very large but connected via flexible hydrophilic linker) to the C-termini of the transmembrane domains of the SNAREs. The work also reports that placing, without linkers, large protein tags at the C-termini of SNAREs on both fusing membranes inhibits content mixing but not lipid mixing. Application of chlorpromazine lifted the block. The paper also presents results of coarse grained simulation analysis convincingly arguing against "penetration model" for w.t C-terminus and, especially, for C-terminus with a small peptide tag. My main concern is that some of the conclusions are not supported by sufficient evidence.

Specific critiques.
1) While the work seems to focus on testing and rejecting the "penetration model" by showing that placing hydrophilic tags on the C-terminus does not prevent content, the title of the work ("Steric hindrance of SNARE transmembrane domain movements impairs the hemifusion-to-fusion transition") suggests the focus on steric hindrance. However this part is not sufficiently developed in the paper. While the interpretation is feasible and illustrated by molecular simulation in Fig 9, there is no analysis (neither experimental nor theoretical) of how bulky the tag has to be to interfere with fusion. The title offers even more specific interpretation by suggesting that bulky tags interfere with the movement of TMDs. Is there any evidence for this conclusion? Do the authors consider chlorpromazine (CPZ) rescue as such evidence?

2) The subtitle of the Subsection "Hemifusion arrest depends on physical proximity of the protein tag to the TMD. " is not justified because the subsection does not have any fusion assays. This section has to have both lipid mixing and complete fusion assays rather than non-quantitative discussion of the vacuole morphology.

3) The subsection describing fusion rescue by CPZ raises many questions. The phrase "We hypothesized that CPZ might promote the hemifusion-to-fusion transition ..." suggests that the authors are unaware of quite a few, perhaps a dozen, papers describing application of CPZ to promote fusion. CPZ has been also used to rescue fusion between yeast vacuoles (Fratti et al., JBC, 282,14861-14867). CPZ has been shown to specifically promote the hemifusion-to-fusion transition (for instance, Melikyan et al., 1997, J Cell Biol. 1997; 136, 995; Chernomordik et al., JCB, 1998, 140, 1369). Note that the interpretation of CPZ promotion in the current paper (via CPZ induced increase in membrane "fluidity", illustrated by FRAP measurements) is different from that in the Melikyan' and Chernomordik' papers (partitioning of the "inverted cone-shaped" CPZ into the inner leaflets of the membranes coming together in hemifusion). The authors need evidence for their interpretation of CPZ promotion. For instance, will any other treatments giving comparable increase in coefficient of lateral diffusion rescue fusion? Also, is there any detectable change in lateral diffusion coefficient for transmembrane SNAREs with and without protein tag?

4) "We could attach small HA or myc tags to vacuolar SNAREs, thereby adding up to five charged residues to the C-terminus, without causing significant effects on fusion." Measuring fusion rates with and without these tags rather than only fusion extents and measuring lipid mixing would significantly strengthen this important conclusion.

5) "This suggests that mixing of the cytoplasmic leaflets may be less dependent on a collective perturbation of lipid structure by SNARE TMDs than the rearrangement of the lumenal leaflets. It is consistent with theory and simulations on the energetics of SNARE-driven fusion, which suggested that fusion pore opening is limited by a larger free energy barrier than the induction of hemifusion [34]. " "Overall, our results are quite compatible with the results and conclusions from recent simulations on SNARE-driven membrane fusion [34]. " Yes, but these findings are also consistent with many earlier studies including papers from labs of Shin and Rothman describing proteoliposome hemifusion by lipid-anchored SNAREs.

6) In Fig. 6D, what is the difference between 3rd and 4th pairs of bars (both labeled as +ATP)?

7) Fig. 3. "C) In vivo vacuole morphology was analyzed as in Fig. 3A. " ; Fig. 6 "B) Vacuole morphology was assessed for the indicated cells as in Fig. 3A." ;Fig. 7B "Vacuole morphology was assessed as in Fig. 3A" Unclear what all these references mean since Fig 3A is just a schematic view of the constructs.

8) The Subsection "Simultaneous C-terminal tagging of Q- and R-SNAREs with large protein domains blocks fusion but not SNARE activation" does not show fusion block. While as noted in this section "vacuole fragmentation is often due to deficiencies in vacuole fusion", the evidence for fusion inhibition is shown only in the next subsection.

Minor:
Ref 45 seems to be a wrong reference for the phrase "Vacuoles carrying both NYV1-EGFP and VAM3-mCitrine in the same membrane [45]"

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The authors present a very well controlled study, which combines biophysical modeling with experimental testing of membrane fusion models. The authors use the appropriate fusion controls, a well established fusion system and the corresponding in vivo controls. The study lacks, however, several controls, quantifications, and explanations, which need to be included.

1) The authors begin with the introduction of the two models, the penetration vs. the indentation model. These need to be explained in figure 1 to make clear what they are aiming at. I would simply see the problem in the full zippering of the SNARE complexes, if they add luminal tags, thus the fusion arrest. Also the discussion of the fusion pore is in my view the result of electrophysiological measurements, likely due to local leakage of membranes. As they can release this block by local membrane perturbance, it shows the crosstalk between lipids and SNAREs in fusion, as implied by Wickner and colleagues in their reconstitution assays (Zick et al, elife 2014). This aspect could be included in the discussion.

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Referee #3:

This manuscript by (D'Agostino, Risselada and Mayer) proposes to unravel the role of the C-termini of SNARE proteins in fusion pore opening, and notably to test two recent models: the perforation model and the indentation model. The perforation model suggests that the C-termini of SNARE proteins are pulled into the hydrophobic core of the lipid bilayer structure during SNARE zippering, which perturbs the stalk intermediate and induces fusion pore opening (Lindau et al. Biophys J

2012). The indentation model proposes that the C-termini of SNARE proteins exert a point-like force on the inner leaflets of the membranes, which generates strong local curvatures and facilitates inner leaflet mixing and thus fusion pore opening. The authors propose to test these two models using a combination of in silico molecular dynamics simulations and in vitro SNARE-mediated fusion experiments between genetically modified vacuoles. Their simulations show that the force exerted by the vacuolar SNARE complex via its C-termini onto the stalk structure is about 18 pN, whereas a force of 80 pN is required for the C-termini to penetrate the membrane. Fusion assays between vacuoles expressing SNAREs carrying linkers and tags of various sizes at their C-termini show that small tags do not affect fusion, whereas large tags (EGFP or mCherry protein tags) allow hemifusion but not full fusion (but see concerns below); fusion is restored when a long linker is added between the transmembrane domain of SNAREs and the protein tag or when only one of the two SNARE partners carry a protein tag. Based on these results, the authors conclude that their work support the indentation model and the notion that several SNARE TMDs cooperate to induce local deformation of inner leaflets, thereby triggering fusion pore opening. I found several issues in the manuscript that the authors might want to consider before publishing this work.

Major concerns
1) Simulation work: I found the first paragraph of the result section difficult to follow and to understand. This probably mainly comes from mislabeling of the associated figures and the rather limited description of the figure legends (this is true for most figure legends of the paper). In Fig. 2A (called Fig. 2B in the text), what is the origin of distance? The method section says that simulations started at 4.8 nm, but the x-axis of Fig. 2A is between 8.4 nm and 7.2 nm. In Fig. 2B (called Fig. 2A in the text), what are the distances indicated in the bilayers? Are they distances between C-termini? If this is the case, why are these distances equivalent for the "wild-type" and the "short peptide" panel? What is the arrow supposed to show? Overall this section would really benefit from significant re-writing to better state the hypothesis to be tested and how the simulations were performed and analyzed for the different conditions wild-type vs. mutants (e.g. how were the energies derived and the forces deduced?).

2) In vitro vacuole fusion assay: the authors should indicate how fusion was quantified from Fig. 3E. Looking at this figure it is not obvious to conclude that 60% of the vacuoles have fused. Could the authors also indicate by arrows in the figure what they consider as fused particles?
Similar vacuole fusion experiments/quantifications should be performed for mutants carrying protein tags in order to conclude that fusion is inhibited when both SNAREs have a protein tag and restored when only one of the two SNAREs carry a protein tag. In the section "Simultaneous C-terminal tagging of Q- and R-SNAREs with large protein domains blocks fusion but not SNARE activation", it seems like the authors only used the vacuolar morphology as an indicator for fusion. This is not sufficient in my opinion.

3) Mutants and assays to probe for hemifusion vs. full fusion: it is not clear to me (but I might have missed something) why the authors used a strain lacking NYV1 and having tagged VAM3. Why not using vacuoles with tagged NYV1 and/or tagged VAM3 as indicated in the legend of Fig. 6C? Similar to my comment above, lipid and content mixing experiments should be performed for mutants with long linkers in order to conclude that full fusion is restored in this case (the authors only look at the rescue of vacuolar morphology in Fig. 7B to draw their conclusion).

4) Perforation vs. indentation model: I agree with the authors that their data do not support the perforation model (in the case of vacuolar fusion) but I do not understand why they claim that it supports the notion that SNARE TMDs cooperate to deform the membranes and mediate fusion pore opening (as proposed by the indentation model). Their data are not in contradiction with the indentation model but I would not say that they support or confirm this model; thus, I think that the conclusions should be softened (or better justified) and the abstract modified accordingly. In addition, it should be mentioned in the abstract that the two models are tested in the context of vacuole fusion (as mentioned at the end of the introduction and discussed on page 10). Regulated exocytosis most likely uses different mechanisms than vacuole fusion; so it is dangerous to draw universal conclusions using a single model.

Other comments:
1) Page 2 - "Vti1"; is it a Qb SNARE?

2) Fig. 1 - the legend should be improved/developed to explain the various stages observed in the context of the 2 models. Is (iii) the dimple phase? For clarity, the hydrophobic mid-plane of the bilayers should be indicated in the figure.
3) Page 5 - "both fluorescent variants of ALP". Does it stand for "protein alkaline phosphatase"?
4) Fig. 3 (and in other figure legends) - "In vivo vacuole morphology was analyzed as in Fig. 3A". Fig. 3A does not show this.
5) Fig. 5C - y-axis = tagged Vam3 and Nyv1.
6) Fig. 6C - What is the difference between wt+V and V+wt experiments? Indicate the purpose of using a Triton control.
7) Fig. 6D, lower panel - What is the condition on the right? With anti-Vam3?
8) Fig. 8 - What is the bleaching geometry? Knowing this geometry is required to deduce the diffusion coefficient.
9) Page 11 - "Our observations suggest (...)". Do you mean "Our simulations"?
10) Page 12 - "(...) only in the presence of a short peptide tag". Do you mean "short linker"?

1st Revision - authors' response 17 June 2016

Response to the referees' comments

We thank all three referees for their careful and constructive evaluation of our manuscript. Their comments have led to inclusion of stronger experimental support, a more precise line of argumentation and a more balanced discussion of our results. The changes that we made in response to the referees' comments are detailed below.

Referee #1:

This study explores mechanisms by which SNAREs mediate fusion of yeast vacuoles. The work presents interesting experimental findings. The authors report that, in contrast to fusion in chromaffin cells, yeast vacuole fusion does not appear to be very sensitive to addition of hydrophilic tags (small or very large but connected via flexible hydrophilic linker) to the C-termini of the transmembrane domains of the SNAREs. The work also reports that placing, without linkers, large protein tags at the C-termini of SNAREs on both fusing membranes inhibits content mixing but not lipid mixing. Application of chlorpromazine lifted the block. The paper also presents results of coarse grained simulation analysis convincingly arguing against "penetration model" for w.t C-terminus and, especially, for C-terminus with a small peptide tag. My main concern is that some of the conclusions are not supported by sufficient evidence.

Specific critiques.
1) While the work seems to focus on testing and rejecting the "penetration model" by showing that placing hydrophilic tags on the C-terminus does not prevent content, the title of the work ("Steric hindrance of SNARE transmembrane domain movements impairs the hemifusion-to-fusion transition") suggests the focus on steric hindrance. However this part is not sufficiently developed in the paper. While the interpretation is feasible and illustrated by molecular simulation in Fig 9, there is no analysis (neither experimental nor theoretical) of how bulky the tag has to be to interfere with fusion. The title offers even more specific interpretation by suggesting that bulky tags interfere with the movement of TMDs. Is there any evidence for this conclusion? Do the authors consider chlorpromazine (CPZ) rescue as such evidence?

We agree with the reviewer that the title did not perfectly match the conclusions that can be drawn from the data. We have changed it into "*Steric hindrance of SNARE transmembrane domain organisation impairs the hemifusion-to-fusion transition"*, which relates better to the experiments shown. With the CPZ experiment we intended only to provide additional evidence that C-terminally tagged SNAREs block the fusion reaction to the hemifusion stage. We did not take it as evidence for TMD movements.

2) The subtitle of the Subsection "Hemifusion arrest depends on physical proximity of the protein tag to the TMD. " is not justified because the subsection does not have any fusion assays. This section has to have both lipid mixing and complete fusion assays rather than non-quantitative discussion of the vacuole morphology.

We have now quantified the vacuole morphology phenotype and added in vitro fusion assays. Similarly as described for the experiment in Fig. 3E, a long peptide spacer connecting the SNARE C-termini to the bulky fluorescent protein is proteolytically cleaved in the lumen of vacuoles that are proteolytically competent (DKY6281). By contrast, the bulky fluorescent protein tag protects short spacers from proteolysis, presumably by sterically hindering the access of vacuolar proteases to it. Proteolytic attack of the long spacers precludes to perform our standard content mixing assay with vacuoles carrying them. Therefore, we had to assay in vitro fusion with proteolytically incompetent vacuoles, which can be done with the microscopy assay used already in Fig. 3E. The results of this in vitro assay confirmed our initial conclusion that SNAREs with the long spacers are functional. Given that complete fusion is directly observed by microscopy, assay of lipid mixing might provide additional information about fusion kinetics, but this would not change our main conclusion that is relevant for the purpose of this manuscript, i.e. that fusion goes to completion when bulky tags are attached via long spacers.

3) The subsection describing fusion rescue by CPZ raises many questions. The phrase "We hypothesized that CPZ might promote the hemifusion-to-fusion transition ..." suggests that the authors are unaware of quite a few, perhaps a dozen, papers describing application of CPZ to promote fusion. CPZ has been also used to rescue fusion between yeast vacuoles (Fratti et al., JBC, 282,14861-14867). CPZ has been shown to specifically promote the hemifusion-to-fusion transition (for instance, Melikyan et al., 1997, J Cell Biol. 1997; 136, 995; Chernomordik et al., JCB, 1998, 140, 1369). Note that the interpretation of CPZ promotion in the current paper (via CPZ induced increase in membrane "fluidity", illustrated by FRAP measurements) is different from that in the Melikyan' and Chernomordik' papers (partitioning of the "inverted cone-shaped" CPZ into the inner leaflets of the membranes coming together in hemifusion). The authors need evidence for their interpretation of CPZ promotion. For instance, will any other treatments giving comparable increase in coefficient of lateral diffusion rescue fusion? Also, is there any detectable change in lateral diffusion coefficient for transmembrane SNAREs with and without protein tag?

The expression we had chosen here was certainly far from optimal. We are, of course, aware of the large body of work that established the fusion-promoting effect of CPZ and we fully agree with its interpretation in terms of membrane curvature. The doubt that we had was whether CPZ would enter living yeast and reach the vacuole (which is not a given; they exclude many drugs other cells are permeable to). The sole intention of the FRAP experiment was hence to test whether CPZ arrived at the vacuole in a living yeast cell. Our purpose was not to propose that CPZ promotes fusion via membrane fluidity. We have re-written this section to make it clearer.
We have also added a control, showing that in cells lacking the SNARE Vam3 CPZ does not rescue vacuole fusion in vivo (Fig. 8E).

4) "We could attach small HA or myc tags to vacuolar SNAREs, thereby adding up to five charged residues to the C-terminus, without causing significant effects on fusion." Measuring fusion rates with and without these tags rather than only fusion extents and measuring lipid mixing would significantly strengthen this important conclusion.

As explained above, proteolytic sensitivity of the lumenal small peptide tags forces us to assess full fusion by microscopy, which allows to use proteolytically incompetent vacuoles, rather than by the standard content mixing assay. Kinetics is difficult to measure in the microscopic assay because of the time it takes to manipulate and transfer the vacuoles to the microscope. Therefore, we capitalize on our experience that, under the conditions we employ, vacuole fusion in test tubes proceeds in a fairly linear fashion up to the 60 min time point (for examples, see JCB 162,211, Fig. 8A or JCB 16,87, Fig. 2C), when we draw the sample for microscopic analysis. Thus, the activity measured at this point correlates well with the fusion rate. Again, we feel that adding lipid mixing data would not add to the argument because since fusion goes to completion anyways.

Since we realized that it is necessary to explain this approach better, we have re-phrased the paragraph describing this experiment and added a paragraph to the methods section on the microscopic assessment of fusion.

5) "This suggests that mixing of the cytoplasmic leaflets may be less dependent on a collective perturbation of lipid structure by SNARE TMDs than the rearrangement of the lumenal leaflets. It is consistent with theory and simulations on the energetics of SNARE-driven fusion, which suggested that fusion pore opening is limited by a larger free energy barrier than the induction of hemifusion [34]. " "Overall, our results are quite compatible with the results and conclusions from recent simulations on SNARE-driven membrane fusion [34]. " Yes, but these findings are also consistent with many earlier studies including papers from labs of Shin and Rothman describing proteoliposome hemifusion by lipid-anchored SNAREs.

This criticism is justified. It is appropriate to cite other studies in this context, which we now do in the revised manuscript.

6) In Fig. 6D, what is the difference between 3rd and 4th pairs of bars (both labeled as +ATP)?

Part of the label of the last bar had been cut off in the pdf version. The 3rd pair of bars provides values "+ATP" and the 4th pair shows "+ATP +anti-Vam3". This last pair, where we added a limited amount of antibodies to the SNARE Vam3, serves as an additional negative control.

7) Fig. 3. "C) In vivo vacuole morphology was analyzed as in Fig. 3A. " ; Fig. 6 "B) Vacuole morphology was assessed for the indicated cells as in Fig. 3A." ;Fig. 7B "Vacuole morphology was assessed as in Fig. 3A" Unclear what all these references mean since Fig 3A is just a schematic view of the constructs.

Thank you for bringing up these errors of labeling, which we have corrected.

8) The Subsection "Simultaneous C-terminal tagging of Q- and R-SNAREs with large protein domains blocks fusion but not SNARE activation" does not show fusion block. While as noted in this section "vacuole fragmentation is often due to deficiencies in vacuole fusion", the evidence for fusion inhibition is shown only in the next subsection.

We have re-organized the subsections and adapted their titles and think that this resolved the issue.

Minor:
Ref 45 seems to be a wrong reference for the phrase "Vacuoles carrying both NYV1-EGFP and VAM3-mCitrine in the same membrane [45]"

Indeed, there is no reference needed at this point. We have removed it.

"iii) hemifusion diaphragm, [51] fusion pore." Should it be "iii) hemifusion diaphragm, iv) fusion pore."?

Yes, for sure. We have corrected this.

Referee #2:

D'Agostino et al. address in their study the role of SNARE transmembrane domains in membrane fusion. Models suggest that the TMDs may either go through a penetration step during the hemifusion to fusion transition or follow an indentation model of cooperation by local deformation of the luminal leaflets. Using the vacuole fusion system, the authors address this issue by adding large domains to the C-termini of the Q (Vam3) and R-SNARE (Nyv1). Their data show that one tag can be tolerated, two result in a fusion deficiency. This can be rescued by either extending the distance between TMD and the luminal fusion protein, thus

minimizing interference or by increasing the fluidity of membranes with the help of the drug chlorpromazine.

The authors present a very well controlled study, which combines biophysical modeling with experimental testing of membrane fusion models. The authors use the appropriate fusion controls, a well established fusion system and the corresponding in vivo controls. The study lacks, however, several controls, quantifications, and explanations, which need to be included.

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The revised Results section now begins with a much more extensive description of the models. Our simulations did not yield any hint that the addition of lumenal tags would be incompatible with SNARE zippering, which is why we did not invoke partial zippering as a potential explanation for the effects. However, we recognize that this is a point to be addressed and now comment on it in the discussion.

We did not understand the context of the referee's phrase *"Also the discussion of the fusion pore is in my view the result of electrophysiological measurements, likely due to local leakage of membranes."* Concerning the last point of the referee, the crosstalk of lipids and proteins: Since our study does not address the crosstalk between SNAREs and non-bilayer lipids in fusion, we feel that a section of the discussion dedicated to this point might distract the reader from the focus of the manuscript. However, we now mention the fusion-promoting effect of non-bilayer lipids in the relevant paragraph of the Results section, where the effect of CPZ is described.

2) What is the difference between Figure 4A and 5A? To me they say the same. They could be combined. The authors should include a quantification of the observed fragmentation.

Figures 4A and 5A are not equivalent. Figure 4A mainly provides an important control showing that the fluorescently tagged SNAREs still localize to vacuoles, which is crucial for interpreting their effects. By contrast, Figure 5A aimed to define vacuolar morphology via a vacuolar lipid probe, which yields much more homogeneous staining than fluorescent SNAREs and allows to assess vacuole fragmentation more reliably. Therefore, we prefer to keep both figures. In accord with the reviewer's suggestion, we now provide quantification of vacuole morphology in Fig. 5A.

3) Likewise in Figure 7B, quantification of the phenotypes should be included.

The quantification has been added.

4) Figure 8E contains an in vivo scoring of the vacuole morphology phenotype before and after addition of the drug CPZ. Their pictures are hard to appreciate. They should show wild-type and mutant cells in parallel and extend their time course. How sensitive are cells to the CPZ addition? If the phenotype is reversed after such a short period, it indeed suggests a fusion arrest. Is the same seen, if the authors use vam3 deletion vacuoles instead? I would not expect this, but it would be a good control for their assay.

We agree with reviewer and we included other two controls (WT and vam3Δ), extending the time course up to 20 minutes. As shown in new Figure 8E, CPZ efficiently rescued in-vivo vacuole fusion even after the first time point of 5 minute whereas in vam3Δ cells, CPZ did not produce any fusion event even after a longer time of incubation (20 minutes). WT cells remained fairly unaffected by CPZ, confirming the in-vitro result (Fig. 8D).

Referee #3:

This manuscript by (D'Agostino, Risselada and Mayer) proposes to unravel the role of the C-termini of SNARE proteins in fusion pore opening, and notably to test two recent models: the perforation model and the indentation model. The perforation model suggests that the C-termini of SNARE proteins are pulled into the hydrophobic core of the lipid bilayer structure during SNARE zippering, which perturbs the stalk intermediate and induces fusion pore opening (Lindau et al. Biophys J 2012). The indentation model proposes that the C-termini of SNARE proteins exert a point-like force on the inner leaflets of the membranes, which generates strong local curvatures and facilitates inner leaflet mixing and thus fusion pore opening. The authors propose to test these two models using a combination of in silico molecular dynamics simulations and in vitro SNARE-mediated fusion experiments between genetically modified vacuoles. Their simulations show that the force exerted by the vacuolar SNARE complex via its C-termini onto the stalk structure is about 18 pN, whereas a force of 80 pN is required for the C-termini to penetrate the membrane. Fusion assays between vacuoles expressing SNAREs carrying linkers and tags of various sizes at their C-termini show that small tags do not affect fusion, whereas large tags (EGFP or mCherry protein tags) allow hemifusion but not full fusion (but see concerns below); fusion is restored when a long linker is added between the transmembrane domain of SNAREs and the protein tag or when only one of the two SNARE partners carry a protein tag. Based on these results, the authors conclude that their work support the indentation model and the notion that several SNARE TMDs cooperate to induce local deformation of inner leaflets, thereby triggering fusion pore opening. I found several issues in the manuscript that the authors might want to consider before publishing this work.

Major concerns
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We agree with the deficiencies outlined by the referee and have completely rewritten the simulation part and extended the figure legends.

Some additional explanations: The 8.4 nm and 7.2 nm corresponds to the thickness of the initially formed stalk-like intermediate. This thickness is defined as the distance between the C-termini. This distance is highly relevant because it determines the stage of SNARE zipping and the mechanical energy stored within the SNARE complex. At this stage, i.e. in the stage of hemifusion, the force which the SNARE complex exerts is expected to reach its maximal value. It is expected that these forces will decrease when the stalk expands because this relaxes the bent SNARE helices. Evidently, exerting force on a stalk intermediate leads to indentation and progression of the fusion pore in our simulations (Fig. 1). 'Perforation' is a property which mainly relates to the chemical nature of the membrane (the process is extremely localized and only involves few lipids). Therefore, analogous to the work of Lindau et al. [39], we studied this scenario in a normal, planar model (POPC) membrane. The noted 4.8 nm is related to the membrane setup used to determine the perforation regime. To avoid misunderstanding, we revised these figures and defined the quantity 'relative indentation'. It is a negative number because it reflects the depth of the 'wells' formed within the membrane upon squeezing. It is defined as the distance over which the c-termini have performed active work to indent the membrane. The slope of the distance-work plot defines the force on the C-termini. We used the same relationship to define the SNARE forces – we pull the C-termini to derive forces. However, here the trick is to additionally calculate the force/work required

to form a similar indentation in the presence of unstructured SNARE linkers (unbiased work) – unstructured linkers impair the ability of the SNARE to perform work. The actual mechanical work performed by the SNARE complex is then this unbiased work minus the work performed in the presence of structured linkers.

2) In vitro vacuole fusion assay: the authors should indicate how fusion was quantified from Fig. 3E. Looking at this figure it is not obvious to conclude that 60% of the vacuoles have fused. Could the authors also indicate by arrows in the figure what they consider as fused particles?

The in-vitro vacuole fusion was assessed by mixing in the in-vitro fusion reaction the two different classes of vacuoles expressing alkaline phosphatase (Pho8) carrying GFP or mCherry tags. Fusion was not quantified by counting vacuoles but by determining the degree of co-localization between the two marker proteins by an unbiased algorithm, which we found to be a simpler and very robust method. Nevertheless, we have now added arrows to indicate what is scored as fused vacuoles, although quantitation of our fusion assay does not require a morphological evaluation. We have added a description of the microscopic fusion assay to the Methods section that clearly states the principle of this assay.

Similar vacuole fusion experiments/quantifications should be performed for mutants carrying protein tags in order to conclude that fusion is inhibited when both SNAREs have a protein tag and restored when only one of the two SNAREs carry a protein tag.

We do show quantitative data for this in Figure 6C, which demonstrates that fusion is inhibited only if bulky protein tags are on both sides of a trans-SNARE complex and that it proceeds if only one side carries the tag. We have now also added quantifications of the microscopic fusion assays showing that small peptide tags permit fusion in vitro (Fig. 3F) and that in vitro (Figs. 7D,E) and in vivo (Figs 7B,C) fusion can be restored for bulky tags if they are detached from the SNARE C-termini by a sufficiently long spacer.

In the section "Simultaneous C-terminal tagging of Q- and R-SNAREs with large protein domains blocks fusion but not SNARE activation", it seems like the authors only used the vacuolar morphology as an indicator for fusion. This is not sufficient in my opinion.

We agree with the reviewer and changed the subtitle to "*Bulky protein domains at the C-termini of Q- and R-SNAREs allow SNARE activation*". In addition, we have included a quantification of vacuole morphology. Inhibition of fusion is now mentioned only in the subsequent section, which provides the necessary data to support it.

3) Mutants and assays to probe for hemifusion vs. full fusion: it is not clear to me (but I might have missed something) why the authors used a strain lacking NYV1 and having tagged VAM3. Why not using vacuoles with tagged NYV1 and/or tagged VAM3 as indicated in the legend of Fig. 6C?

This is necessary because vacuoles are a homotypic fusion system, i.e., by default, both fusion partners carry the same SNAREs. By consequence, trans-SNARE complexes can form in both orientations. In order to assure that trans-SNARE complexes could form only in one orientation and that only trans- but not cis-SNARE complexes could simultaneously carry two bulky tags, we removed Nyv1 from one fusion partner, an modification that leaves the vacuoles fusogenic (Nichols et al., 1997 and Fig. 6C). This operation makes the interpretation straightforward. We have added an illustration of the setup to Fig. 6C

Similar to my comment above, lipid and content mixing experiments should be performed for mutants with long linkers in order to conclude that full fusion is restored in this case (the authors only look at the rescue of vacuolar morphology in Fig. 7B to draw their conclusion).

We have performed in vitro fusion experiments for mutants with the long spacers, which are now shown in Fig. 7D and 7E. Since, similar to the peptide tags used in Fig. 3E, the longer spacers are cleaved in proteolytically competent vacuoles, we we resorted to the microscopic in vitro fusion

assay with proteolytically incompetent vacuoles, which leave the linker stable. Full fusion was quantified via the co-localization of two differentially labelled vacuolar membrane proteins, using an algorithm of the ImageJ software package. Given that the long spacers restore full fusion, additional lipid mixing assays would not influence this conclusion.

4) Perforation vs. indentation model: I agree with the authors that their data do not support the perforation model (in the case of vacuolar fusion) but I do not understand why they claim that it supports the notion that SNARE TMDs cooperate to deform the membranes and mediate fusion pore opening (as proposed by the indentation model). Their data are not in contradiction with the indentation model but I would not say that they support or confirm this model; thus, I think that the conclusions should be softened (or better justified) and the abstract modified accordingly. In addition, it should be mentioned in the abstract that the two models are tested in the context of vacuole fusion (as mentioned at the end of the introduction and discussed on page 10). Regulated exocytosis most likely uses different mechanisms than vacuole fusion; so it is dangerous to draw universal conclusions using a single model.

The referee has probably overlooked that the abstract did mention vacuoles as our model system. We have revised the abstract and now explicitly state that we evaluate these two models specifically for vacuolar fusion. We fully agree that – even if when one disproves the perforation/penetration model – this does not necessarily imply that one proves the indentation model. Therefore, we soften our claims by explicitly mentioning that our findings are “consistent with” the indentation model rather than claiming that vacuolar fusion involves indentation. Nevertheless, our simulations suggest that 'indentation' followed by membrane remodeling provides a likely scenario.

Other comments:
1) Page 2 - "Vti1"; is it a Qb SNARE?

Yes, we have added this now.

2) Fig. 1 - the legend should be improved/developed to explain the various stages observed in the context of the 2 models. Is (iii) the dimple phase? For clarity, the hydrophobic mid-plane of the bilayers should be indicated in the figure.

We have now better distinguished the two leaflets and provide a better description in the legend.

3) Page 5 - "both fluorescent variants of ALP". Does it stand for "protein alkaline phosphatase"?

Yes, ALP stands for alkaline phosphatase. We now define this acronym when it occurs the first time in the text.

4) Fig. 3 (and in other figure legends) - "In vivo vacuole morphology was analyzed as in Fig. 3A". Fig. 3A does not show this.

Yes, a consequence of having re-arranged the figures before submission. We have corrected this.

5) Fig. 5C - y-axis = tagged Vam3 and Nyv1.

We have adopted this label, which is more precise.

6) Fig. 6C - What is the difference between wt+V and V+wt experiments? Indicate the purpose of using a Triton control.

For the content mixing assay we use vacuoles from two different strain backgrounds that are mixed in vitro. One contributes pro-alkaline phosphatase (BJ3505) and the other one the respective maturase (DKY6281). The difference between wt+V and V+wt is that the tagged Vam3 is once in BJ3505 and in the other case in DKY6281. We have improved the labeling of this figure in order to make this evident. Performing the reaction in the presence of Triton X-100 permeabilizes the vacuoles and permits the maturase access to the pro-alkaline phosphatase in a fusion-independent way. This is a convenient way of verifying that these two reporter enzymes for the assay are present

at the same level in the various mutant combinations tested. We have added a sentence to the figure legend to explain this.

7) Fig. 6D, lower panel - What is the condition on the right? With anti-Vam3?

Antibodies to Vam3 are a common means to inhibit trans-SNARE pairing and fusion, providing an additional negative control and demonstrating that the observed signal is SNARE-dependent. We added a phrase to the figure legend to express this clearly.

8) Fig. 8 - What is the bleaching geometry? Knowing this geometry is required to deduce the diffusion coefficient.

We agree. Since we have not determined this geometry precisely we removed the estimated diffusion coefficients from the figure. The only purpose of this experiment was to demonstrate that CPZ reaches the vacuoles in living yeast cells, for which a qualitative CPZ-induced increase in recovery from photobleaching is sufficient. The data provide this qualitative difference.

9) Page 11 - "Our observations suggest (...)". Do you mean "Our simulations"?

Yes. We have changed the phrase accordingly.

10) Page 12 - "(...) only in the presence of a short peptide tag". Do you mean "short linker"?

We realized that our nomenclature was potentially confusing and have changed it throughout the manuscript. We now reserve "linker" for the linker region between SNARE domains and the SNARE TMDs. The sequences that separate bulky proteins from the SNARE C-terminus are referred to as "spacer" and the myc and HA tags used are referred to as peptide tags. In addition, we now provide the type of spacer whenever a construct is mentioned. We feel that this enhances the clarity.

2nd Editorial Decision 11 July 2016

Thank you for the submission of your research manuscript to our editorial offices. We have now received the enclosed reports on it. As you will see, while all referees in principle support publication of the study in EMBO reports, reviewer 3 raises one point that would need to be addressed experimentally before publication. Additional issues raised by this reviewer can be addressed in writing.

Given the overall positive assessment of the referees, I would like to give you the opportunity to address the remaining issues raised by reviewer 3 and submit the final version to EMBO reports.

REFEREE REPORTS

Referee #1:

Additional data and editing have strengthened the paper and addressed my concerns. I recommend publication.

Referee #2:

The authors addressed my questions with additional experiments. The added controls strengthen the manuscript. I have no further requests.

Referee #3:

In this revised version, the authors have addressed most of the concerns raised by the reviewers. Their new title, abstract and text now better reflect the conclusions that can be drawn from their data. However I still have some comments that should be addressed to make the story both stronger and easier to follow.

Main comments:

I thank the author for explaining 1) their simulation work and 2) why they could not always use their standard content-mixing assay (and thus used instead a microscopy-based content-mixing assay) and for indicating in Fig. 3E and 7D what they consider as fused vacuoles. It is still difficult from such pictures to appreciate that the amount of co-localization in fact corresponds to that indicated in the associated histogram (especially in the mutant system). In addition, co-localization measured by ImageJ plug-in can sometimes lead to false positive corresponding for example to particles in close proximity (especially in systems such as the one studied here in which particles are massively aggregated and display various sizes). So I think that the author should either perform key lipid mixing experiments (as suggested by reviewer 1 in the first report) - especially since they have the full expertise to do it as shown in Fig. 6D - or (if doable) characterize fusion through changes in vacuole size, providing a complete histogram of vacuole sizes in the various conditions tested.

The nomenclature used to describe the different variants is sometimes very confusing in my opinion, notably in the section "Simultaneous C-terminal tagging of Q- and R-SNAREs in trans blocks the hemifusion to fusion transition" because it does not seem to match with that of Fig. 6 and its legend. In the text, NYV1-S9-EGFP (N) seems to be (NYV1-S9-EGFP VAM3), i.e. a vacuole expressing Vam3 and a fluorescent version of Nyv1. In the legend of Fig. 6, NYV1-S9-EGFP (N) seems to be (ΔNYV1ΔVAM3; NYV1-S9-EGFP), i.e. a vacuole expressing only a fluorescent version of Nyv1 ("(...) strains that were double deleted for Nyv1 and Vam3 (delNV) and expressed either nothing, Nyv1-S9-EGFP (N), Vam3-S9-mCitrine (V), or both.")

I was also confused by the "delN" condition in Fig. 6C (this is not defined anywhere in the text). This condition was not presented in the first version of the manuscript. Is this supposed to stand for the "WT" condition? In fact, several conditions mentioned in the text are missing in Fig. 6C: "Vacuoles carrying NYV1-S9-EGFP (N) fused efficiently with wildtype vacuoles" (i.e. condition N + WT); "(...) giving signals comparable to those of delNV" (i.e. delNV + delNV); "Vacuoles carrying both NYV1-S9-EGFP and VAM3-S9-mCitrine in the same membrane fused efficiently with wild-type organelles" (i.e. NV + WT).

Along the same line, are the N+V and WT+WT conditions in Fig. 6D (content mixing) similar to those of Fig. 6C?

Having the same definitions everywhere and a cartoon of the different conditions would really help (but not like the one in Fig. 6C which uses again another nomenclature).

Other comments:

Use the same nomenclature throughout the text for defining bilayer leaflets: choose amongst "inner", "lumenal" or "cis" and the corresponding term for the other leaflet. I would suggest to use inner/outer leaflets.

Define FP in Fig. 2.

Write "Nyv1Δ Vam3Δ" instead of "ΔΔ" in Fig. 3B.

Explain in the legend of Fig. 3E and 7D what is indicated by arrows.

Fig. 5C (graph on the right): indicate in the y-axis that Nyv1 is a tagged version.

Legend of Fig. 9: Upper panel=short spacer

2nd Revision - authors' response 27 July 2016

Response to the comments of referee #3:

We thank the referee for the attention dedicated to our manuscript and for the suggestions for improving it, which have been very valuable.

Referee's comments (in bold):
In this revised version, the authors have addressed most of the concerns raised by the reviewers. Their new title, abstract and text now better reflect the conclusions that can be drawn from their data. However I still have some comments that should be addressed to make the story both stronger and easier to follow.

Main comments:

I thank the author for explaining 1) their simulation work and 2) why they could not always use their standard content-mixing assay (and thus used instead a microscopy-based content-mixing assay) and for indicating in Fig. 3E and 7D what they consider as fused vacuoles. It is still difficult from such pictures to appreciate that the amount of co-localization in fact corresponds to that indicated in the associated histogram (especially in the mutant system). In addition, co-localization measured by ImageJ plug-in can sometimes lead to false positive corresponding for example to particles in close proximity (especially in systems such as the one studied here in which particles are massively aggregated and display various sizes). So I think that the author should either perform key lipid mixing experiments (as suggested by reviewer 1 in the first report) - especially since they have the full expertise to do it as shown in Fig. 6D - or (if doable) characterize fusion through changes in vacuole size, providing a complete histogram of vacuole sizes in the various conditions tested.

Our response:

Lipid mixing experiments for these strains are doable but, as we had explained in our response to reviewer 1, they would not provide the critical information that is needed in this context, which is whether the fusion pore opens or not. But we agree with this referee that a better justification for our microscopic fusion assay is desirable. Therefore, we have added Figure EV1, which provides elements that this referee has asked for, and some more:

- Fig. EV1A shows the distribution of vacuole size after fusion +- ATP for the wildtype and for the various tagged versions of vacuoles, as demanded by the referee. The results from these measurements are consistent with the results from the fusion assay based on colocalization of fluorescent markers that we had shown in the revised version
- We share the reviewers concern that a colocalization approach to measure fusion could be prone to false positive signals. Therefore, we had tested this before but not included respective data in the manuscript. We now show an experiment indicating that, when both assays are used in parallel on fusing non-tagged vacuoles, the colocalization assay faithfully reproduces signals from the standard content mixing assay (Fig. EV1C). This data validates the colocalization approach.

The nomenclature used to describe the different variants is sometimes very confusing in my opinion, notably in the section "Simultaneous C-terminal tagging of Q- and R-SNAREs in trans blocks the hemifusion to fusion transition" because it does not seem to match with that of Fig. 6 and its legend. In the text, NYV1-S9-EGFP (N) seems to be (NYV1-S9-EGFP VAM3), i.e. a vacuole expressing Vam3 and a fluorescent version of Nyv1. In the legend of Fig. 6, NYV1-S9-EGFP (N) seems to be (ΔNYV1ΔVAM3; NYV1-S9-EGFP), i.e. a vacuole expressing only a fluorescent version of Nyv1 ("(...) strains that were double deleted for Nyv1 and Vam3 (delNV) and expressed either nothing, Nyv1-S9-EGFP (N), Vam3-S9-mCitrine (V), or both.") I was also confused by the "delN" condition in Fig. 6C (this is not defined anywhere in the text). This condition was not presented in the first version of the manuscript. Is this supposed to stand for the "WT" condition? In fact, several conditions mentioned in the text are missing in Fig. 6C:

"Vacuoles carrying NYV1-S9-EGFP (N) fused efficiently with wildtype vacuoles" (i.e. condition N + WT); "(...) giving signals comparable to those of delNV" (i.e. delNV + delNV); "Vacuoles carrying both NYV1-S9-EGFP and VAM3-S9-mCitrine in the same membrane fused efficiently with wild-type organelles" (i.e. NV + WT).

Along the same line, are the N+V and WT+WT conditions in Fig. 6D (content mixing) similar to those of Fig. 6C?

Having the same definitions everywhere and a cartoon of the different conditions would really help (but not like the one in Fig. 6C which uses again another nomenclature).

Yes, we found it difficult to find a good compromise between having all nomenclature in Figure 6 and not making it too complex. The referee's confusion - in fact we had not added new conditions - illustrates that our revised Figure has not been clear. Therefore, we have changed the identifiers of the strains in this figure and now give the exact combination of SNAREs for both fusion partners in each sample. We have also adjusted the nomenclature in the cartoon of Fig. 6C, such that it fits with the one from the neighboring graph. We hope that this now avoids ambiguities.

Other comments:

Use the same nomenclature throughout the text for defining bilayer leaflets: choose amongst "inner", "lumenal" or "cis" and the corresponding term for the other leaflet. I would suggest to use inner/outer leaflets.
We now use "outer" and "inner" leaflet throughout.

Define FP in Fig. 2.
This is now defined

Write "Nyv1Δ Vam3Δ" instead of "ΔΔ" in Fig. 3B.
This has been corrected

Explain in the legend of Fig. 3E and 7D what is indicated by arrows.
This is now explained

Fig. 5C (graph on the right): indicate in the y-axis that Nyv1 is a tagged version.
This is now indicated

Legend of Fig. 9: Upper panel=short spacer
This has been corrected

3rd Editorial Decision 12 August 2016

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

REFEREE REPORT

Referee #3

The authors have fully addressed all of my remaining concerns. I therefore now recommend publication of their study in EMBO Reports.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Andreas Mayer
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2016-42209V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
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  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No exclusion
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For evaluating vacuolar morphology in living cells, photos were taken blindly and random fields in the sample. Results were confirmed by an independent investigator. For measuring fusion activity by microscopy, fusion products were identified in an automated fashion by a co-localization algorithm of the ImageJ software package, which is unbiased.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	They are defined once in the methods section because they are the same for all figures
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
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