JCB

Ultrastructural plasma membrane asymmetries in tension and curvature promote yeast cell fusion

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May 26, 2021

Re: JCB manuscript #202103142

Prof. Sophie G Martin
University of Lausanne
Department of Fundamental Microbiology
Biophore Building
Lausanne CH-1015
Switzerland

Dear Dr. Martin,

Thank you for submitting your manuscript entitled "Ultrastructural plasma membrane asymmetries in tension and curvature promote yeast cell fusion." The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

The editors and reviewers all agree that this is a very interesting and well-executed study that breaks new ground and is highly appropriate for JCB. The large majority of the reviewers' comments would not require new data and could be addressed by text changes. One aspect referred to by reviewers 2 and 3 was the observation that the smooth/wavy and protrusive phenotypes could be observed in both mating types (albeit at different frequency). I wonder whether these are stable traits based on cell "identities" or whether they may fluctuate on rapid timescales so that (for example) a single cell might switch between smooth and wavy plasma membrane, perhaps due to stochastic fluctuations in endo/exocytosis.

The main experimental requests were from reviewer 2, who pointed out the desirability of a larger number of fus1Δ images. This seems worthwhile. The value of looking at fus1Δ/gpd1Δ double mutants is less clear to me, especially in light of reviewer 3's comments on difficulties of interpretation, so I leave it to you to decide on whether or not it would be worth the additional time and effort. I would hope to be able to assess a revised version that addressed the reviewer comments without needing re-review. Thank you for submitting this fascinating study to JCB!

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, https://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior

to publication.

IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

As you may know, the typical timeframe for revisions is three to four months. However, we at JCB realize that the implementation of social distancing and shelter in place measures that limit spread of COVID-19 also pose challenges to scientific researchers. Lab closures especially are preventing scientists from conducting experiments to further their research. Therefore, JCB has waived the revision time limit. We recommend that you reach out to the editors once your lab has reopened to decide on an appropriate time frame for resubmission. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you again for this interesting contribution to Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Daniel Lew, PhD
Monitoring Editor
Journal of Cell Biology

Dan Simon, PhD
Scientific Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

Considering the central role of cell-cell fusion in reproduction, development, and tissue regeneration, this study is interesting, and the experiments are logical and well-performed. The authors reveal asymmetries between the two gametes using 3D CLEM and proceed to show that these asymmetries arise from differences in turgor pressure between the cells and differences in the ratio between membrane addition and membrane removal. The imaging and associated quantifications

are very impressive, providing a never-before-seen view of yeast mating. While the concept of an asymmetric fusion focus ("fusogenic synapse" in drosophila myoblasts) is not entirely new, the findings of the paper are certainly novel and would likely open new research directions in the field. I also think that quantitative EM studies have a lot of merit over the existing, sometimes anecdotal, data. I only have minor comments that might further improve the manuscript.

Minor comments:

- The authors state that "Fusogenic protein... likely drive the formation of fusion pore(s), though this has not been directly observed in any system". I found this to be misleading as if to suggest that maybe fusogenic proteins do not drive the formation of pores, which has been shown for several viral fusogenic protein as well as for the EFF-1 fusogenic protein in C. elegnans. Direct observation is not necessarily a pre-requisite to define protein function. Since EFF-1 and HAP-2 have clear structural homology to viral fusogenic proteins I would say it is more than likely that they drive the formation of pores.
- The organelles from P and M cells will inevitably mix after fusion and this mixing may depend on a form of regulated trafficking. Perhaps the authors can address this in the discussion and put it in the context of what they observe. The authors mention in several places that the fusion focus "excludes other organelles" but the observation should not be misinterpreted as to suggest that the organelles don't eventually mix or that the only post-fusion event is karyogamy.
- Along similar lines, is it also possible that organelles fragment/vesiculate prior to fusion? Such fragmentation might facilitate regulated mixing of the cellular organelles. I am not familiar enough with organelle structure in yeast but I wonder if the authors care to speculate. It seems like something one could even test by fluorescence microscopy but I am not sure about how easy this would be in yeast given their small size and it is anyway well beyond the scope of the current manuscript. It might interest the authors to know that in fusing mouse myoblasts both Golgi and ER are fragmented and this is not well known or heavily studied but evidence does exist in literature.
- The authors might also compare and contrast their observation with Chlamydomonas gamete fusion where one of the fusing partners is also more invasive/protrusive than the other.

Reviewer #2 (Comments to the Authors (Required)):

Cell fusion is important for many fundamental processes in cell and developmental biology including sexual reproduction, skeletal muscle formation, and bone remodeling. In this study, the authors use CLEM to define the detailed morphological changes of the mating partners at the contact site in fission yeast. The authors made several important observations. They found that the "fusion focus" contains linear actin filaments and two distinct population of vesicles that likely represent exocytic and endocytic vesicles, respectively. Other organelles appear to be excluded from the fusion focus. They also defined the process of fusion pore initiation and expansion at the ultrastructural level for the first time. Surprisingly, the authors also found that the mating partners, traditionally considered isogametes, are engaged in the fusion process via asymmetric structures at several levels. The M (or h-) cell forms a protrusive structure that pushes into the P (h+) cell during the cell-cell contact phase, especially during the PM contact phase. The PM is smooth in M cells and wavy in P cells. In addition, the exocytosis/endocytosis ratio, as indicated by the ratio of Myo52/Fim1, is higher in P cells than in M cells. Such an analysis at the ultrastructural level is both timely and foundational for mechanistic analysis of cell-cell fusion in fission yeast. The discovery of the asymmetric structure-mediated cell fusion also makes the fission yeast model globally relevant to other cell-cell fusion systems.

That being said, there are a few weaknesses that need to be addressed:

1. The structural asymmetry with respect to the mating types: while the protrusive structure is preferentially formed by the M cell, the reverse asymmetry (i.e., the P cell forms the protrusive structure) also occurs at a non-trivial frequency. How can this happen theoretically? Does this also happen in other cell fusion systems? For example, in terms of myoblast-myotube fusion, is the podosome-like structure always formed by the myoblast? Can a myotube also form a podosome-like structure at certain frequency that penetrates into a myoblast? This issue needs to be discussed.

2. The force behind the protrusive structural formation: the authors presented nice data indicating the "involvement" of turgor pressure (regulated by Gpd1) in the protrusive structural formation and the requirement of Gpd1 for promoting fusion efficiency in M over P cell. However, turgor pressure is unlikely to be the main mechanism. In the case of myoblast-myotube formation, the protrusive structure is actin-based. In the case of fission yeast mating, the protrusion is likely driven by concerted actions of an actin-based structure (the actin fusion focus?), cell-wall weakening (which allows turgor pressure to drive membrane protrusion), and turgor pressure. The authors also implied that Fus1 is involved in the protrusion formation. If so, it would be interesting and important to test how gpd1Δ and fus1Δ double deletions affect the protrusive structural formation, mating, and fusion efficiency. Alternatively, the turgor pressure could be differentially regulated during cell fusion in different mating types. For example, the turgor pressure could be stronger in M cells than in P cells. If so, the localized breakdown of cell wall at the contact site would allow the turgor pressure in the M cell to drive protrusion formation.

3. The role of Fus1 in membrane asymmetry and cell fusion: the authors presented clear data indicating that deletion of FUS1 preferentially reduces exocytic vesicles and affects fusion efficiency in P cells. However, the role of Fus1 in membrane morphology (smooth vs. wavy and convex vs. concave/flat) is less clear. This is mainly caused by the small number of fus1Δ cells examined by CLEM. While recognizing that CLEM is technically challenging and time-consuming, perhaps the authors should do a few more cells to solidify their conclusion.

4. Exo-endocytic dynamics during cell fusion: theoretically, during the initial stage of cell-cell fusion, endocytosis should prevail as there is a net membrane loss during the fusion pore formation. The authors presented data (Figure S2A) suggesting that the endocytic vesicles are slightly more than the exocytic vesicles during the far-CW-contact stage, but not the subsequent 3 stages. One would have expected endocytosis to dominate during the PM-contact stage, not at the far-CW-contact stage. Thus, it would be helpful to explain and discuss this issue.

5. The title of the manuscript: perhaps it is better to delete "in tension and curvature".

6. In the Abstract: "abrogates" and "prevents" are a little too strong.

Reviewer #3 (Comments to the Authors (Required)):

This interesting paper takes a close look at the structure of the site of cell fusion during mating in the fission yeast S. pombe, using state of the art correlative light and electron microscopy (CLEM). The use of tomography allows an unprecedented level of detail in the investigation of cell fusion

site, and provides important information about the membranes, and actin cables at the "fusion focus". The overall observations confirm earlier findings about the dense concentration of vesicles in this area, based on FP-tagged proteins in S. pombe and EM from the budding yeast S. cerevisiae. However, the use of tomography provided several important new contributions. First, capturing most or all of the fusion pore suggests that initiation may occur at more than one site, or might progress non-uniformly. Second, and more surprising, the two mating types were seen to have different membrane ultrastructures, with P-cells having a more ruffled appearance, possibly due to increased exocytosis and less endocytosis than M-cells. Differences in turgor pressure are further thought to influence the protrusion of the M-cell into the P-cell. Previously yeast cell mating was thought to be symmetric at the level of the mechanism of cell fusion, whereas higher cells, such as Drosophila myoblast fusion are seen to be highly asymmetric. These observations suggest that yeast cell fusion may also be intrinsically asymmetric, in line with some earlier findings concerning the kinetics of fusion focus formation.

On the whole this manuscript reflects a very careful, detailed and thorough analysis of the fusion site ultrastructure, and provides important new insights in to the events of cell fusion in this excellent model organism. The authors make a good case for correlation of the various phenotypes with mating type (P cells show more ruffles, M cells protrude into P cells more, P cells have more exocytotic vesicles relative to endocytosis, the fus- mutation has a stronger effect on M cells, opposite effects of gpd-).

Less clear to this reviewer is whether the ultrastructural differences are causative (i.e. the protruding membrane is required for, or, drives fusion; membrane waviness somehow prepares the membrane for fusion), or is the secondary result of some other causative effect. Although the authors try to distinguish causation from effect using mutants, the effect of mutations in the formin, fus1, is far too crude an instrument, given the major effects on the fusion focus and possibly of other actin-dependent processes. Similarly, the gpd1- mutations could cause all manner of regulatory effects on the osmotic and cell wall integrity pathways, which might confound a clear conclusion. Indeed, as the authors conclude, the differences cannot be entirely necessary or even genetically determined, as they observed cells in which the pattern was reversed. Moreover, there are examples of relatively weak correlations; on page 6 it is stated: "78% (18/23) of h+ cells, but only 30% (7/23) of h- cells, had wPM". This does not seem to be the level of correlation one would expect from a mechanistically required asymmetry. I could not help but wonder if the asymmetries are not simply a consequence of the previously reported difference in timing for the establishment of the fusion focus in P and M cells. Perhaps M cells soften up their cells walls earlier and more uniformly than P cells allowing for a smoother margin and protrusion due to small differences in turgor pressure. Perhaps P cells are still removing cell wall and so the membrane is wavier because it is still doing exocytosis that the M cells have already finished. Or perhaps the cell wall is removed in a patchy and non-homogenous way such that the plasma membrane is simply following the margin of a partially eroded cell wall.

In sum, although the data is quite lovely and interesting, this reviewer did not find the interpretations compelling, and other interpretations should be considered. While there does appear to be some kind of asymmetry between the M and P cells in S. pombe cell fusion, the authors are overstating or are misleading about the results in budding yeast. Although the observation that there are some wavy membranes in images from Brizzio et al., (1996) the lack of knowledge about mating type makes it impossible to identify the cause of these asymmetries as due to mating type, and not differences in timing or turgor pressure. But more significantly, the observation that the fps1 mutation has a somewhat different effect in the two mating types (stated on page 12), seems to be much less important than the observation that mating between two fps1 mutants largely

restored the cell fusion defect (Philips and Herskowitz, 1997). That is, balanced turgor pressures is more important than differential pressures for allowing cell fusion. I note that this also seems to be true in the S. pombe gpd1 x gpd1 mating, but given that in one mating-type gpd1- cells fuse better than wild-type, it is hard to interpret the outcome. Finally, the authors have neglected a relative recent paper (J Cell Biol 2017 Dec 4;216(12):3971-3980. doi: 10.1083/jcb.201703169) in which fps1- was used to unbalance the turgor pressure between the two mating cells, leading to a block in cell fusion when it was in either mating type and balanced turgor pressure restored cell fusion. In that paper, the effect appeared to be regulatory, which would tend to weaken the argument that protrusion is necessary in a mechanistic sense. But budding yeast may be different, so the observations in that paper may simply serve to contrast the two species. Either way, it should be discussed.

Minor points:

The authors should use either h+ and h- or P and M consistently in the text and figures. It is very confusing for readers to have to go back and forth.

Figure 3 C-E - I found the green models of the cell fusion site to be very hard to interpret until I saw the video in the supplementary data. Without more information it is hard to know how the model corresponds to the tomographic slices. This requires more description or some visual aid to indicate how the orientation of the slices with respect to the models. This is partly because the model in C shows what look to be ling linear fusion pores, which is counter-intuitive.

At the bottom of page 7 it says: "Neither Myo52 nor Fim1 levels were significantly different between mating types (Fig 6E-F). This is not shown anywhere in Figure 6. In fact, the impression is that they are different, unless one goes deep into the figure legend to see that the measurements are actually of the maximum intensity at the cell fusion site. Since this is a crucial control, it should be shown.

We thank the reviewers for their careful reading of our work and their interesting comments. We respond below to each of the comments.

Reviewer #1 (Comments to the Authors (Required)):

Considering the central role of cell-cell fusion in reproduction, development, and tissue regeneration, this study is interesting, and the experiments are logical and well-performed. The authors reveal asymmetries between the two gametes using 3D CLEM and proceed to show that these asymmetries arise from differences in turgor pressure between the cells and differences in the ratio between membrane addition and membrane removal. The imaging and associated quantifications are very impressive, providing a never-before-seen view of yeast mating. While the concept of an asymmetric fusion focus ("fusogenic synapse" in drosophila myoblasts) is not entirely new, the findings of the paper are certainly novel and would likely open new research directions in the field. I also think that quantitative EM studies have a lot of merit over the existing, sometimes anecdotal, data. I only have minor comments that might further improve the manuscript.

Minor comments:

- The authors state that "Fusogenic protein... likely drive the formation of fusion pore(s), though this has not been directly observed in any system". I found this to be misleading as if to suggest that maybe fusogenic proteins do not drive the formation of pores, which has been shown for several viral fusogenic protein as well as for the EFF-1 fusogenic protein in C. elegnans. Direct observation is not necessarily a pre-requisite to define protein function. Since EFF-1 and HAP-2 have clear structural homology to viral fusogenic proteins I would say it is more than likely that they drive the formation of pores.

We have corrected the wording, which was indeed misleading. It was not our intention to doubt the role of fusogenic proteins, but simply to state that fusion pore formation and expansion has not been directly observed for cell-cell fusion.

- The organelles from P and M cells will inevitably mix after fusion and this mixing may depend on a form of regulated trafficking. Perhaps the authors can address this in the discussion and put it in the context of what they observe. The authors mention in several places that the fusion focus "excludes other organelles" but the observation should not be misinterpreted as to suggest that the organelles don't eventually mix or that the only post-fusion event is karyogamy.

Organelles from the two gametes likely mix to some extent in the zygote (though for instance mitochondria do not; see Chako et al, JCB 2019). Because we have not studied this directly here, we prefer to abstain from direct comment. To clarify when the exclusion of other organelles from the fusion focus occurs, we have now specified that the fusion focus structure is only present from about 1h

before fusion to 10 min post-fusion and that the fusion pore keeps expanding after it disassembles.

- Along similar lines, is it also possible that organelles fragment/vesiculate prior to fusion? Such fragmentation might facilitate regulated mixing of the cellular organelles. I am not familiar enough with organelle structure in yeast but I wonder if the authors care to speculate. It seems like something one could even test by fluorescence microscopy but I am not sure about how easy this would be in yeast given their small size and it is anyway well beyond the scope of the current manuscript. It might interest the authors to know that in fusing mouse myoblasts both Golgi and ER are fragmented and this is not well known or heavily studied but evidence does exist in literature.

Thank you for this interesting comment. We have not investigated whether organelles fragment, though it is interesting that we never observed Golgi stacks in our tomograms. As Golgi exists as stacks in mitotically growing cells, it is possible there is some fragmentation during sexual reproduction. Because this is not a central observation to our work, and because we have not probed it further, we prefer not to directly comment on this idea at this point.

- The authors might also compare and contrast their observation with Chlamydomonas gamete fusion where one of the fusing partners is also more invasive/protrusive than the other.

Indeed, this is a good point. We have added mention of the *Chlamydomonas* asymmetric mating structures in the introduction.

Reviewer #2 (Comments to the Authors (Required)):

Cell fusion is important for many fundamental processes in cell and developmental biology including sexual reproduction, skeletal muscle formation, and bone remodeling. In this study, the authors use CLEM to define the detailed morphological changes of the mating partners at the contact site in fission yeast. The authors made several important observations. They found that the "fusion focus" contains linear actin filaments and two distinct population of vesicles that likely represent exocytic and endocytic vesicles, respectively. Other organelles appear to be excluded from the fusion focus. They also defined the process of fusion pore initiation and expansion at the ultrastructural level for the first time. Surprisingly, the authors also found that the mating partners, traditionally considered isogametes, are engaged in the fusion process via asymmetric structures at several levels. The M (or h-) cell forms a protrusive structure that pushes into the P (h+) cell during the cell-cell contact phase, especially during the PM contact phase. The PM is smooth in M cells and wavy in P cells. In addition, the exocytosis/endocytosis ratio, as indicated by the ratio of Myo52/Fim1, is higher in P cells than in M cells. Such an analysis at the ultrastructural level is both timely and foundational for mechanistic analysis of cell-cell fusion in fission yeast. The discovery of the asymmetric structure-mediated cell fusion also makes the fission yeast model globally relevant to other

cell-cell fusion systems.

That being said, there are a few weaknesses that need to be addressed:

1. The structural asymmetry with respect to the mating types: while the protrusive structure is preferentially formed by the M cell, the reverse asymmetry (i.e., the P cell forms the protrusive structure) also occurs at a non-trivial frequency. How can this happen theoretically? Does this also happen in other cell fusion systems? For example, in terms of myoblast-myotube fusion, is the podosome-like structure always formed by the myoblast? Can a myotube also form a podosome-like structure at certain frequency that penetrates into a myoblast? This issue needs to be discussed.

This is an interesting point. We do not know at this stage how the asymmetries between P- and M-cells are established. There is clearly one element linked to the cell identity, as the average distribution, whether tested by electron microscopy or light microscopy, show a skew linked to mating type. It could be, as also suggested by the Editor, that single cells rapidly switch between the two phenotypes due to fluctuations in the rate of exocytosis, but with a longer average time spent in the smooth (for the M-cell) or wavy (for the P-cell) configuration. This could explain our findings of some WT cell pairs exhibiting reverse configuration relative to the majority. Another possibility is that the asymmetries arise from a small difference between the two cell types, with stochastic noise that may occasionally reverse the directionality of the difference. One could imagine for instance that pressure difference between the two cell types is sensed mechanically at the cell-cell contact and becomes amplified by signaling through the cell wall integrity pathway. We have expanded the discussion to present these options.

Regarding other fusion system, as we have not worked on them directly, we cannot comment on the penetrance of the protrusion directionality. In Sens et al 2010 describing podosomes in the *Drosophila* myoblast-myotube system, the authors state that from light-microscopy images, 35% of fusion structures appeared as protrusions that invaded the founder cell (myotube), but how the 65% others are organized is not stated. We could not find quantitative information on the electron microscopy.

2. The force behind the protrusive structural formation: the authors presented nice data indicating the "involvement" of turgor pressure (regulated by Gpd1) in the protrusive structural formation and the requirement of Gpd1 for promoting fusion efficiency in M over P cell. However, turgor pressure is unlikely to be the main mechanism. In the case of myoblast-myotube formation, the protrusive structure is actin-based. In the case of fission yeast mating, the protrusion is likely driven by concerted actions of an actin-based structure (the actin fusion focus?), cell-wall weakening (which allows turgor pressure to drive membrane protrusion), and turgor pressure. The authors also implied that Fus1 is involved in the protrusion formation. If so, it would be interesting and important to test how gpd1Δ and fus1Δ double deletions affect the protrusive structural

formation, mating, and fusion efficiency. Alternatively, the turgor pressure could be differentially regulated during cell fusion in different mating types. For example, the turgor pressure could be stronger in M cells than in P cells. If so, the localized breakdown of cell wall at the contact site would allow the turgor pressure in the M cell to drive protrusion formation.

Our data indeed point to a difference in turgor pressure between the two partner cells, with the M-cell showing higher pressure and thus protrusion into its partner. We have clarified this in the discussion. As pointed out by the reviewer, for turgor pressure to produce a protrusion, the cell wall likely needs to be weakened, for which Fus1 contributes by concentrating the delivery of vesicles containing cell wall digestive enzymes at the cell-cell contact site. Our now extended *fus1*Δ dataset indeed shows that h- *fus1Δ* cells are less likely to protrude into their partner. Whether actin assembly directly produces a protrusive force is less clear. We have not examined the ultrastructure of the fusion site in *gpd1Δ fus1Δ* double mutants, as we are not sure how informative the results would be. Because protrusion is strongly reduced in both mutants, it will likely also be strongly reduced in the double mutant.

3. The role of Fus1 in membrane asymmetry and cell fusion: the authors presented clear data indicating that deletion of FUS1 preferentially reduces exocytic vesicles and affects fusion efficiency in P cells. However, the role of Fus1 in membrane morphology (smooth vs. wavy and convex vs. concave/flat) is less clear. This is mainly caused by the small number of fus1Δ cells examined by CLEM. While recognizing that CLEM is technically challenging and time-consuming, perhaps the authors should do a few more cells to solidify their conclusion.

We have acquired 13 new *h+ fus1Δ x h- WT* (now 27 in total) and 11 new *h+ WT x h- fus1Δ* (now 20 in total) tomograms to consolidate the *fus1Δ* data and interpretation. This extended dataset confirms our findings that the number of secretory vesicles is reduced in *fus1Δ* and that the plasma membrane of the mutant is smoother. To quantify this phenotype more stringently, we have now used a quantitative method, described in the methods section, where we measured both amplitude and wavelength of plasma membrane waves and defined a cut-off to attribute the cells to the smooth or wavy category. We applied this method throughout the manuscript, which led to minor changes in numbers throughout, but no change in the overall results. The results clearly show that while about 70% of WT *h+* cells exhibit a wavy PM, only 1 in 27 *h+ fus1Δ* cells does so. These data thus confirm the link between membrane waviness and local secretion.

Our larger dataset also shows that deletion of *fus1* in the *h-* cell compromises the protrusion activity of this cell. Indeed, membrane curvature in *h+ WT x h- fus1Δ* pairs was as likely to be convex for the *h+* as the *h-* cell and almost no protrusion was observed. By contrast, in *h+ fus1Δ x h- WT* pairs, the *h-* WT was seen to protrude into its partner. We conclude that both the actin fusion focus and sufficient turgor pressure are required for cell protrusion.

4. Exo-endocytic dynamics during cell fusion: theoretically, during the initial stage of cell-cell fusion, endocytosis should prevail as there is a net membrane loss during the fusion pore formation. The authors presented data (Figure S2A) suggesting that the endocytic vesicles are slightly more than the exocytic vesicles during the far-CW-contact stage, but not the subsequent 3 stages. One would have expected endocytosis to dominate during the PM-contact stage, not at the far-CW-contact stage. Thus, it would be helpful to explain and discuss this issue.

Over the entire fusion process, where one can assume the cell surface goes from two hemispheres to one cylinder, the net membrane surface is constant (two hemisphere surface = $2*2\pi r^2 = 4\pi r^2$; one cylinder surface of height $2r = 2\pi r * 2r = 4\pi r^2$). How this balance is predicted to evolve over the course of the fusion process is less clear. We agree with the reviewer that a temporary increase in endocytosis at the time of fusion pore expansion could be predicted. This may be a very transient stage that we do not capture in our measurements. We also note that our measurements are of number of vesicles, which may not give a direct estimate of rates.

5. The title of the manuscript: perhaps it is better to delete "in tension and curvature".

We would prefer to leave the title as it is, as it conveys a clearer message than if it just states “asymmetries” without further precision. The ultrastructural information clearly reveals differences in both membrane tension and curvature between the two partner cells.

6. In the Abstract: "abrogates" and "prevents" are a little too strong.

We replaced “abrogates” with “strongly diminishes” (concerning the effect of *fus1Δ* on membrane waviness) and “prevents” with “impedes” (concerning the effect of *gpd1Δ* on cell protrusion).

Reviewer #3 (Comments to the Authors (Required)):

This interesting paper takes a close look at the structure of the site of cell fusion during mating in the fission yeast S. pombe, using state of the art correlative light and electron microscopy (CLEM). The use of tomography allows an unprecedented level of detail in the investigation of cell fusion site, and provides important information about the membranes, and actin cables at the "fusion focus". The overall observations confirm earlier findings about the dense concentration of vesicles in this area, based on FP-tagged proteins in S. pombe and EM from the budding yeast S. cerevisiae. However, the use of tomography provided several important new contributions. First, capturing most or all of the fusion pore suggests that initiation may occur at more than one site, or might

progress non-uniformly. Second, and more surprising, the two mating types were seen to have different membrane ultrastructures, with P-cells having a more ruffled appearance, possibly due to increased exocytosis and less endocytosis than M-cells. Differences in turgor pressure are further thought to influence the protrusion of the M-cell into the P-cell. Previously yeast cell mating was thought to be symmetric at the level of the mechanism of cell fusion, whereas higher cells, such as Drosophila myoblast fusion are seen to be highly asymmetric. These observations suggest that yeast cell fusion may also be intrinsically asymmetric, in line with some earlier findings concerning the kinetics of fusion focus formation.

On the whole this manuscript reflects a very careful, detailed and thorough analysis of the fusion site ultrastructure, and provides important new insights in to the events of cell fusion in this excellent model organism. The authors make a good case for correlation of the various phenotypes with mating type (P cells show more ruffles, M cells protrude into P cells more, P cells have more exocytotic vesicles relative to endocytosis, the fus- mutation has a stronger effect on M cells, opposite effects of gpd-).

Less clear to this reviewer is whether the ultrastructural differences are causative (i.e. the protruding membrane is required for, or, drives fusion; membrane waviness somehow prepares the membrane for fusion), or is the secondary result of some other causative effect. Although the authors try to distinguish causation from effect using mutants, the effect of mutations in the formin, fus1, is far too crude an instrument, given the major effects on the fusion focus and possibly of other actin-dependent processes. Similarly, the gpd1- mutations could cause all manner of regulatory effects on the osmotic and cell wall integrity pathways, which might confound a clear conclusion. Indeed, as the authors conclude, the differences cannot be entirely necessary or even genetically determined, as they observed cells in which the pattern was reversed. Moreover, there are examples of relatively weak correlations; on page 6 it is stated: "78% (18/23) of h+ cells, but only 30% (7/23) of h- cells, had wPM". This does not seem to be the level of correlation one would expect from a mechanistically required asymmetry. I could not help but wonder if the asymmetries are not simply a consequence of the previously reported difference in timing for the establishment of the fusion focus in P and M cells. Perhaps M cells soften up their cells walls earlier and more uniformly than P cells allowing for a smoother margin and protrusion due to small differences in turgor pressure. Perhaps P cells are still removing cell wall and so the membrane is wavier because it is still doing exocytosis that the M cells have already finished. Or perhaps the cell wall is removed in a patchy and non-homogenous way such that the plasma membrane is simply following the margin of a partially eroded cell wall.

We agree with the reviewer. It is very difficult to know to what extent the observed asymmetries are functionally helping the fusion process. The interpretation is especially difficult in the case of *fus1Δ*, as its main role is to promote cell wall degradation by concentrating the delivery of digestive enzyme-containing secretory vesicles. In fact, any mutation reducing secretion is

predicted to affect both cell wall digestion and plasma membrane waviness, such that the two effects may not be uncoupled. We have been very careful in our wording, as also pointed out by the reviewer, to explain these difficulties in interpretation. It remains intriguing that *h+ fus1Δ* show a significantly more severe phenotype than *h- fus1Δ* cells. We have now modified the discussion to be even clearer about the difficulty in interpreting the *fus1Δ* phenotype and removed the proposed causality in the abstract.

Regarding the *gpd1Δ* phenotype, we feel that the interpretation is easier. Because deleting *gpd1* in both partner cells restores fusion efficiency to wildtype levels, we can conclude that the differences in fusion efficiency observed when it is deleted only in one of the two partners are due to relative changes between the two cells rather than overall loss of a pathway important for cell fusion. The effect of *gpd1Δ* on turgor pressure has been measured in vegetative cells, so we are quite confident that the deletion leads to turgor pressure reduction. Of course, we cannot exclude that *gpd1Δ* leads to other signaling changes that would have opposite effect in the two cell types, but the most conservative interpretation, which also fits with the protrusion observations, is that the cause is a change in turgor pressure.

In sum, although the data is quite lovely and interesting, this reviewer did not find the interpretations compelling, and other interpretations should be considered. While there does appear to be some kind of asymmetry between the M and P cells in S. pombe cell fusion, the authors are overstating or are misleading about the results in budding yeast. Although the observation that there are some wavy membranes in images from Brizzio et al., (1996) the lack of knowledge about mating type makes it impossible to identify the cause of these asymmetries as due to mating type, and not differences in timing or turgor pressure. But more significantly, the observation that the fps1 mutation has a somewhat different effect in the two mating types (stated on page 12), seems to be much less important than the observation that mating between two fps1 mutants largely restored the cell fusion defect (Philips and Herskowitz, 1997). That is, balanced turgor pressures is more important than differential pressures for allowing cell fusion. I note that this also seems to be true in the S. pombe gpd1 x gpd1 mating, but given that in one mating-type gpd1- cells fuse better than wild-type, it is hard to interpret the outcome. Finally, the authors have neglected a relative recent paper (J Cell Biol 2017 Dec 4;216(12):3971-3980. doi: 10.1083/jcb.201703169) in which fps1- was used to unbalance the turgor pressure between the two mating cells, leading to a block in cell fusion when it was in either mating type and balanced turgor pressure restored cell fusion. In that paper, the effect appeared to be regulatory, which would tend to weaken the argument that protrusion is necessary in a mechanistic sense. But budding yeast may be different, so the observations in that paper may simply serve to contrast the two species. Either way, it should be discussed.

We are sorry if we gave the feeling that we were mis-interpreting the *S. cerevisiae* literature. Regarding the observation of wavy membranes in Brizzio et al., (1996), we agree that neither the cause nor the cell type can be identified, but given the previous observations noted in Baba et al (1989), this feature is likely

to be present in *S. cerevisiae*. We have now removed citation of Brizzio et al (1996), as this point is not discussed in that paper.

The *fps1Δ* data in *S. cerevisiae* is very clear that deletion of this regulator in both cells re-establishes fusion competency, and we indeed made a similar observation with *gpd1Δ* in S. pombe. The question is whether these genetic manipulations reveal that turgor pressure is (and has to be) equal in the two partner cells, or whether there may be underlying differences in turgor pressure independent of these regulators. In *S. pombe*, our observation of protrusion from the M-cell and absence thereof when that cell lacks *gpd1* is a clear indication that this cell normally has a stronger pressure than the P-cell. In unpublished data, we also found that in *prm1Δ* cell pairs, which are incompetent at plasma membrane fusion, the M-cell forms a directional bubble of cytosol into the P-cell, in agreement with the idea that the M-cell exhibits stronger turgor pressure.

We agree that the *S. cerevisiae* literature suggests that the two cells exhibit more equal pressure. Though, to our knowledge, there is no large ultrastructural dataset during cell fusion in *S. cerevisiae*, light microscopy in Smith et al, JCB 1997 (cited by the reviewer above) shows a similar effect on curvature of the cell-cell contact site upon *fps1* deletion in either cell. Similarly, observations of cytosol bubbles in the *prm1Δ* mutant are reported to happen at similar frequency in both directions (Heiman et al, JCB 2000). Thus, it may be that turgor pressure regulation is different in the two species. We have revised the discussion to make this point and cite these papers.

Minor points:

The authors should use either h+ and h- or P and M consistently in the text and figures. It is very confusing for readers to have to go back and forth.

We had tried to use *h+* and *h-* for all genetic notation and P and M when speaking about the cell type more generally, thinking this was actually easier. If this adds confusion, we are happy to homogenize our notation. We have now used *h+* and *h-* throughout.

Figure 3 C-E - I found the green models of the cell fusion site to be very hard to interpret until I saw the video in the supplementary data. Without more information it is hard to know how the model corresponds to the tomographic slices. This requires more description or some visual aid to indicate how the orientation of the slices with respect to the models. This is partly because the model in C shows what look to be ling linear fusion pores, which is counter-intuitive.

We have changed the figure legend to improve the description of the position of the tomographic z-slices on the model representation to help with interpretation. We realize that this is not easy, which is exactly why we provide the supplementary videos.

At the bottom of page 7 it says: "Neither Myo52 nor Fim1 levels were significantly different between mating types (Fig 6E-F). This is not shown anywhere in Figure 6. In fact, the impression is that they are different, unless one goes deep into the figure legend to see that the measurements are actually of the maximum intensity at the cell fusion site. Since this is a crucial control, it should be shown.

We are sorry if the text was unclear. We measured Myo52 and Fim1 signals at the fusion site, with values reported both as fluorescence profile and average peak intensity (over 5 pixels). Myo52 and Fim1 fluorescence values are significantly different between mating types in wildtype cells (shown in Fig 5E-F), but not in *fus1Δ* (shown in Fig 6E-F). The sentence in question lacked "in *fus1Δ* cells", which we have now added.

July 19, 2021

RE: JCB Manuscript #202103142R

Prof. Sophie G Martin
University of Lausanne
Department of Fundamental Microbiology
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Switzerland

Dear Prof. Martin:

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